U.S. DEPARTMENT OF COMMERCE
National Technical Information Service

PB-270 910

Detection and Inactivation of Enteric Viruses in Wastewater

Hadassah Medical School, Jerusalem (Israel)

Prepared for

Environmental Monitoring and Support Lab -Cincinnati, Ohio Biological Methods Branch

May 77

PB 270 210

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

[illegible]

[illegible]

1. Environmental Health Effects Research
2. Environmental Protection Technology
3. Ecological Research
4. Environmental Monitoring
5. Socioeconomic Environmental Studies
6. Scientific and Technical Assessment Reports (STAR)
7. Interagency Energy-Environment Research and Development
8. "Special" Reports
9. Miscellaneous Reports

[illegible]

[illegible]

TECHNICAL REPORT DATA

(Please read Instructions on the reverse before completing)

1. REPORT NO.
EPA-600/2-77-095

2.

3. RECIPIENT'S ACCESSION NO.

4. TITLE AND SUBTITLE
DETECTION AND INACTIVATION OF ENTERIC VIRUSES IN WASTEWATER

5. REPORT DATE
May 1977 issuing date

6. PERFORMING ORGANIZATION CODE
""

7. AUTHOR(S)
Hillel I. Shuval and Eliyahu Katzenelson

8. PERFORMING ORGANIZATION REPORT NO.
""

9. PERFORMING ORGANIZATION NAME AND ADDRESS
Environmental Health Laboratory
Hebrew University-Hadassah Medical School
Jerusalem, Israel

10. PROGRAM ELEMENT NO.
1BD713

11. CONTRACT/GRANT NO.
S-800990
(formerly 17060EAM)

12. SPONSORING AGENCY NAME AND ADDRESS
Environmental Monitoring and Support Lab. - Cin., OH
Office of Research and Development
U.S. Environmental Protection Agency
Cincinnati, Ohio 45268

13. TYPE OF REPORT AND PERIOD COVERED
Final (Oct. 1969-Jan. 1975)

14. SPONSORING AGENCY CODE
EPA/600/06

15. SUPPLEMENTARY NOTES

16. ABSTRACT

This report covers studies on the development and evaluation of methods for concentrating and assaying low levels of viruses in large volumes of water as well as studies on the use of ozone in inactivating viruses in water and wastewater.

Of the eight virus concentration methods evaluated, filtration with cellulose nitrate membranes, aluminum hydroxide and PE-60 proved most promising. The feasibility of using hollow fiber membranes was demonstrated. A rapid method capable of detecting viruses in water in less than 24 hours using fluorescent antibodies was developed. A spectrophotometric method of detecting low concentrations of ozone in small (10 ml) samples of water was developed. Kinetic studies show that ozone inactivates enteroviruses more rapidly than chlorine under comparable conditions. With a 0.3 ppm residual ozone inactivates 99% of seeded poliovirus in clean water in less than 10 seconds as compared to 100 seconds for chlorine. Although no detectable dose-response relationship could be demonstrated for ozone contact times greater than 10 seconds, preliminary studies indicate that such a realtionship may exist for shorter contact times. The kinetic curve of virus inactivation having a rapid first stage lasting seconds followed by a slower stage lasting minutes may be associated with virus clumping phenomenon. Ozone has been shown to kill viruses rapidly and effectively in wastewater effluent.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Viruses,* water, wastewater, public health, filtration, ozone, disinfection, water quality	Concentration detection, inactivation monitoring, methods, Israel	06M

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS (This Report)	21. NO. OF PAGES
Release to public	Unclassified	30[illegible]
	20. SECURITY CLASS (This page)	22. PRICE
	Unclassified	PC A14 | MF A01

EPA Form 2220-1 (9-73)

/ a

EPA-600/2-77-096
May 1977

DETECTION AND INACTIVATION OF ENTERIC VIRUSES IN WASTEWATER

by

Hillel I. Shuval
Eliyahu Katzenelson
Environmental Health Laboratory
Hebrew University-Hadassah Medical School
Jerusalem, Israel

Grant No. S-800990
(formerly 17060 EAM)

Project Officer

Gerald Berg
Biological Methods Branch
Environmental Monitoring and Support Laboratory
Cincinnati, Ohio 45268

ENVIRONMENTAL MONITORING AND SUPPORT LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
CINCINNATI, OHIO 45268

DISCLAIMER

This report has been reviewed by the Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the U.S. Environmental Protection Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

FOREWORD

Man and his environment must be protected from the adverse effects of pesticides, radiation, pathogens, noise, and other forms of pollution, and the unwise management of solid waste. Efforts to protect the environment require a focus that recognizes the interplay between the components of our physical environment--air, water, and land. The Environmental Monitoring and Support Laboratory-Cincinnati contributes to this multidisciplinary focus through programs engaged in

- studies on the effects of environmental contaminants on the biosphere, and
- a search for ways to prevent contamination and to recycle valuable resources.

The viruses that are discharged into sewage with the fecal wastes of man, and thereby into the environment, constitute a hazard to all of those who contact the waters that are insulted by the presence of these viruses. Methodology that is necessary to detect and quantify small numbers of these highly infective disease-producing agents in large volumes of water, and methodology that is necessary to destroy them with ozone are described in this report.

Dwight G. Ballinger
Director
EMSL-Cincinnati

ABSTRACT

This report covers studies on the development and evaluation of methods for concentrating and detecting low levels of viruses in large volumes of water. In addition, it covers studies on the use of ozone in inactivating viruses in water and wastewater.

Eight recognized virus concentration methods were evaluated under standardized conditions and their virus recovery efficiency was determined. Of particular interest are the methods which proved efficient and also capable of concentrating viruses from water samples of 100 gallons or more. The most promising methods in this category are filtration methods using cellulose nitrate membranes, aluminum hydroxide and PE-60. A new method developed and evaluated in the course of the study used hollow fiber membranes. Viruses from large volumes of water can be concentrated rapidly by this method without requiring pH adjustment.

A promising method for the rapid detection of viruses in water using the fluorescent antibody technique was developed. This method can provide qualitative results in 6 to 9 hours and a quantitative estimate of virus concentration in 18 hours.

Although much work remains to be done in developing and evaluating virus concentration and detection methods in water, there is good evidence that practical methods for the virus assay of water and wastewater are an achievable goal.

In studies on the use of ozone as a virucidal agent in water and wastewater, special techniques for investigating this question were developed, among them an accurate spectrophotometric method for detecting very low concentrations of ozone in small samples (10 ml) of water. A 0.3 ppm residual of ozone was found to inactivate over 99% of seeded poliovirus in clean water in less than 10 seconds as compared to 100 seconds required to achieve the same degree of inactivation by chlorine under equal conditions.

The kinetic curves of virus inactivation indicates a rapid first stage kill in a matter of seconds followed by a slower kill lasting minutes, until complete inactivation is achieved. The role of virus clumps in explaining this phenomenon is considered.

Although no detectable dose response relationship could be demonstrated for ozone contact times greater than 10 seconds, preliminary studies indicate that such a relation may exist for shorter contact times. Ozone was also shown to kill viruses rapidly and effectively in wastewater effluent. The virucidal effect of various possible ozone species was investigated and has led to some preliminary hypotheses on this subject. Ozone has been shown to be a rapid and effective virucidal agent of potentially great value in field applications in the treatment of water and wastewater.

This report was submitted in May 1975 in fulfillment of Research Grant No. S-800990 (formerly 17060 EAM) under the sponsorship of the U.S. Environmental Protection Agency. This report covers a period from October 1969 to January 1975 and work was complete as of January 1975.

CONTENTS

		Page
Abstract		iv
List of Figures		viii
List of Tables		xi
Acknowledgments		xiv
I	Introduction	1
II	Conclusions	27
III	Recommendations	36

PART A Virus Detection Methods

IV	Review of Methods for the Detection of Enteric Viruses in the Water Environment	42
V	Comparison of Eight Concentration Methods for Isolation of Viruses in Water	69
VI	Virus Concentration using Hollow Fiber Membranes	99
VII	Evaluation of Gauze Pad Method to Recover Viruses from Water	117
VIII	A Rapid Fluorescent Antibody Method for Quantitative Isolation of Viruses from Water	144
IX	Virus Types in Israel Sewage	166

PART B Inactivation of Viruses in Water

X	The Chemistry of Ozone as a Disinfectant	177
XI	Spectrophotometric Method for the Determination of Ozone in Aqueous Solutions	199
XII	Inactivation Kinetics of Viruses and Bacteria in Water by Ozone	225
XIII	Disinfection of Viruses in Sewage by Ozone	254
XIV	Coliphage Inactivation in Seawater	269
XV	List of Publications	286

Preceding page blank

LIST OF FIGURES

Number		Page
1	Detail of Fiber Unit and System Flow Sheet	101
2	Result of a Typical Virus Concentration Experiment	102
3	Scanning Electron Micrographs of the Cross-Section of the Hollow Fiber Membrane	106
4	Concentration of Virus in Feed Versus Volume of Feed Gauze Sampler	109
5	Gauze Sampler	119
6	Sampler in Operation	120
7	Device for Extracting Liquid for Gauze Pad	123
8	BGM Cells Infected with Poliovirus Type I, Stained with Fluorescent Antibodies; 9 Hours after Infection	150
9	BGM Cells Infected with Poliovirus Type I, Stained with Fluorescent Antibodies; 18 Hours after Infection	150
10	The % of Poliovirus in Daily Grab Sampling vs. Daily Gauze Pad in Sewage by Neutralization Test	171
11	Inactivation of Poliovirus 1 by Ozone after 10 Sec of Various pH values (T=5^{0}C)	190
12	Calibration Curve for Determination of Ozone in Water	204
13	Stability of Color Intensity as Function of Potassium Iodide Concentration	208
14	Ozone Aqueous Solution Stability Versus Time, Temperature and Concentration	217
15	Influence of Stirring Rate on Ozone Stability (at 5^{0}C)	218
16	Kinetics of Ozone Demand of Different Virus Suspensions at 5^{0}C and stirring rate 80 rpm	220
17	Inactivation Kinetics of Poliovirus I by 0.3 ppm Ozone at 5^{0}C	212
18	Inactivation Kinetics of Poliovirus I by 0.8 ppm Ozone at 5^{0}C	233

Number		Page
19	Inactivation Kinetics of Poliovirus I by 1.5 ppm Ozone at 5°C	234
20	Virus Survival Rate with Varying Ozone Concentrations at 0.5 sec and 2.5 sec	235
21	Poliovirus I Survival after 40" of Exposure to Various Concentrations of Ozone	237
22	Inactivation Kinetics of Poliovirus I (118-b) Before and After Ultrasonication by 0.1 ppm Ozone	239
23	Inactivation Kinetics of Coliphate T_2 by Various Concentrations of Ozone at 1°C	242
24	Inactivation Kinetics of E. Coli by Various Concentrations of Ozone at 1°C	243
25	Inactivation Kinetics of Poliovirus I, added in two Portions, at 0 Time and after 56", by 1.5 ppm Ozone	245
26	Correlation between Ozone Concentration and Redox Potential Values	246
27	Inactivation Kinetics of Poliovirus I by 0.4 mg/l initial O_3 Concentration in the Presence of 5% and 10% Filtered Sewage	258
28	Inactivation Kinetics of Poliovirus I by 0.8 mg/l initial O_3 Concentration in the Presence of 5% and 10% Filtered Sewage	259
29	Inactivation Kinetics of Poliovirus I by 1.3 mg/l initial O_3 Concentration in the Presence of 5% and 10% Filtered Sewage	260
30	Inactivation Kinetics of Poliovirus I by 1.8 mg/l initial O_3 Concentration in the Presence of 5% and 10% Filtered Sewage	261
31	Kinetics of Increase in O_3 Residual Concentrations and of Poliovirus I Inactivation During Continuous O_3 Bubbling Through Buffer and Filtered Sewage	263
32	Percent Survival of Poliovirus I after Reaction with Different Initial O_3 Concentrations in 5% and 10% Filtered Sewage (Batch Experiments)	266
33	Inactivation of Coliphage T_2 in Normal and Autoclaved Sea Water	272
34	Inactivation of Coliphage T_2 in Sea Water Containing Different Concentrations of Nutrient Broth	273
35	The Effect of Nutrient Broth on the Anticoliphage Activity of a Mixture of Marine Bacteria	275

Number Page

36 Anticoliphage Activity in Different Isolates of Marine Bacteria 277
37 Different Rates of Coliphage Inactivation by an Isolate of Marine Bacteria 278
38 The Effect of Nutrient Borth on the Activation Capacity of an Isolated Marine Bacteria 279
39 The Inactivation of Coliphage in Preincubated Sea Water 280

TABLES

Number Page

1 The Recovery Efficiency of Four Methods for the Concentration of Seeded Attenuated Poliovirus I at Low Input Levels in Tap Water 78

2 The Recovery Efficiency of Five Methods for the Concentration of Seeded Attenuated Poliovirus I at High Input Levels in Tap Water 79

3 The Recovery Efficiency of Four Methods for the Concentration of Seeded Attenuated Poliovirus I at Various Input Levels in Tap Water 80

4 The Recovery Efficiency of Five Methods for the Concentration of Seeded Echovirus 7 at Various Input Levels in Tap Water 81

5 Recovery Efficiency of Four Methods for the Concentration of Enteroviruses from Sewage-Contaminated Water 84

6 Recovery Efficiency of Four Methods for the Concentration of Enteroviruses from Sewage-Contaminated Water 85

7 Recovery Efficiency of the Gelman Cartridge Filter for the Concentration of Seeded Attenuated Poliovirus I at Various Input Levels in Tap Water 86

8 The Average Recovery of Seeded Enterovirus and the Concentration Factor of Five Methods with Tap Water 89

9 The Average Recovery of Seeded Enterovirus and the Concentration Factor of Four Methods with Tap Water 92

10 Results of Backwash Experiments 103

11 Parametric Results from Curve Fitting 110

12 Results of Virus Concentration Experiments 113

13 A Comparison of Enterovirus Concentration by Two Sampling Methods: Grab (2 Liters) and Pad (24 Hours) 125

Number		Page
14	The Average Percentage of Enterovirus Detected on Gauze Pads Held in Sewage for 1 Day Compared with the Percentage on Gauze Pads Held for 3, 4, and 7 Days	126
15	Comparison Between the Number of Viruses Detected from 3 Gauze Pads Immersed in Parallel in Raw Sewage	128
16	The Effect of Successive Gauze Pad Elutions on Poliovirus I Elution (Tap Water-4 Elutions)	130
17	The Effect of Successive Gauze Pad Elutions on Poliovirus I Elution (Tap Water-5 Elutions)	131
18	The Effect of Successive Gauze Pad Elutions on Enterovirus Recovery (Raw Sewage)	132
19	The Effect of Calf Serum on Elution of Poliovirus I From Gauze Pads (Tap Water)	134
20	The Recovery of the Gauze Pad Sample for Concentration of Poliovirus I (Tap Water)	135
21	The Effect of Sample Volume on Poliovirus I Recovery (Tap Water)	137
22	Titers of Poliovirus I Obtained with the Fluorescent Antibody Method after 9 and 20 Hour Incubation, and with the Plaque Count Method on Plates	152
23	Titers of Poliovirus Type I Obtained with the Fluorescent Antibody Technique and with the Plaque Count Method	153
24	Comparison of the Fluorescent Antibody Technique with the Plaque Count Method for Quantitative Evaluation of Viruses in 5 Liters of Water	155
25	Comparison of the Fluorescent Antibody Technique with the Plaque Count Method for the Quantitative Evaluation of Viruses in 40 Liters of Water	156
26	Virus Types Picked from Sewage at Different Places and Communities in Israel	169
27	Polio and Non-Poliovirus in Grab and Gauze Pad Samples in Kiriat Shemoneh Sewage by Neutralization Test	170
28	Summary of the Kinetics of Ozone Decomposition in Water	183

Number Page

29 Influence of Potassium Iodide Concentration on Intensity of Color 207

30 Reproducibility of Results in the Spectrophotometric Method 210

31 Comparison of Titrimetric and Spectrophotometric Methods for Determination of Ozone in Water 213

ACKNOWLEDGEMENTS

This report represents a concerted effort by a research team over a five year period. Many of the staff members served during the whole period while others participated in specific projects over shorter time periods. It is not possible to list here the names of all those who contributed each in his own way to the success of this project.

The scientists who played major roles in these studies are listed as authors of the sections as follows:

Section I: Hillel I. Shuval
Section IV: Eliyahu Katzenelson & H.I. Shuval
Section V: Badri Fattal, E. Katzenelson, T. Hostovsky & H.I. Shuval
Section VI: Georges Belfort, Y. Rotem-Borensztajn & E. Katzenelson
Section VII: Badri Fattal & E. Katzenelson
Section VIII: Eliyahu Katzenelson
Section IX: Badri Fattal
Section X: Mordechai Peleg
Section XI: Hannah Scherhter
Section XII: Eliyahu Katzenelson, B. Kletter & H.I. Shuval
Section XIII: Eliyahu Katzenelson & N. Biedermann
Section XIV: Binyamin Kletter, E. Katzenelson & H.I. Shuval

In addition mention should be made of the contributions by M. Green, R. Kalbo, J. Sabag, M. Hod, T. Goldblum, M. Nevo and Dr. M. Nishmi. The active cooperation of the Staff of the Ministry of Health and the Mekorot Water Company in carrying out the field sampling programs is sincerely appreciated.

SECTION I

INTRODUCTION

ENTEROVIRUSES AND WATER QUALITY

The ultimate amount of water annually available to a country is more or less fixed in volume. Its rate of development is dependent on the availability of financial resources and engineering technology. Into this fairly constant source of supply is sowed an ever increasing amount of organic and inorganic chemical waste together with pathogenic bacteria and viruses dispensed into the environment by steadily growing populations massing in burgeoning urban centers. This expanding populace requires more and more water for urban and industrial use, but its very own wastes continually contaminate the limited available supply, despoiling its quality. This process reaches, at times, a point where the water's utility for human consumption is at risk. Thus a built-in paradox of modern society revolves around a situation where more and more water is needed by the growing population but less and less becomes available at the required quality as a result of the self-destructive process of pollution. In this section we shall attempt to evaluate the impact of the increasing burden of enteroviruses on water quality and see what should be done about it.

THE GROWING DEMAND FOR WATER

Man's health and well-being are dependent in many ways both on the quantity and the quality of water available to meet his varying needs. For many years it has been accepted that the quality of the water he drinks or uses for cooking or other domestic purposes may have a direct effect on his health. Today we are becoming no less concerned with the burden of pathogenic bacteria and viruses in water used for recreation, agricultural irrigation, or growing shellfish. We are also asking whether pathogens sprayed into the air during wastewater treatment processes or land disposal systems may be carried as aerosols to infect people in the vicinity.

As the population of the world grows at an average rate of 2 percent per year, doubling every 30 years, more and more water must be extracted from rivers, lakes and from the ground to supply the additional people. The extent of this increasing demand for water can be understood in historical terms in light of the statement that the number of people alive today in the world is equal to the total number of persons who ever lived on the face of the earth since the beginning of mankind. Each person uses directly or indirectly from 50 to 500 liters of water per day and returns most of that water to the drainage systems of the earth together with a residual load of inorganic and organic pollutants as well as with a burden of pathogenic microorganisms that are shed together with the human body wastes. These once-used waters, together with their animate and inanimate residuals, return to the water sources supplying the essential fluid of life to those living downstream.

THE LIMITS OF NATURAL SELF-PURIFICATION

In theory there is enough water in the world to dilute all of the domestic wastewater produced by man in a single year by a factor greater than one million. Even the most advanced efforts of science and technology could hardly expect to achieve such a high degree of residual reduction in any controlled waste treatment process.

However, such a hypothetical calculation is highly misleading since it assumes a continuous and uniform distribution of all human wastes into the total volume of water of the world. In fact, 97 percent of the world's water is in the oceans and is generally not available as a direct repository or diluent of the wastes of human population centers. Of the remaining amount of sweet water, three-fourths is locked up in the earth's polar ice caps and glaciers. It has been estimated that only about 0.01 percent of the total water of the globe is actually in our rivers.

The self-purifying powers of the aqueous ecosystem have been depended upon for many generations to rid our streams and rivers of the pollutional load through such processes as dilution, sedimentation, predation and biological decomposition. However, this capacity has its limits and can become severely overstressed as the insult of pollution ever increases.

The problem is even more complex than it might appear due to the social forces which are at work and which are changing the demographic distribution of people on the face of the globe. One hundred years ago 80 percent and more of the population was thinly settled in rural areas while today more and more countries are approaching a situation where 80 percent of the population is crowded together in cities, most of them strung one after the other along the lengths of the rivers of the world.

Today with some 317 billion people in the world, about 30 percent are crowded together in urban areas with central sewage systems disposing of their wastes mainly to the rivers. By the year 2000 we anticipate the total population of the world to double and during the same period the urban population is expected to reach about 60 percent of the total. That means an absolute increase of 400 percent in the urban population with a parallel increase in the pollution burden of domestic wastes, including pathogenic bacteria and viruses in our rivers and other bodies of water serving as repositories of urban wastes.

We are already faced with a situation where some of our rivers are now so loaded with such vast amounts of wastes of all kinds, including human body wastes laden with the full spectrum of enteropathogens, that by the time they reach the sea almost all, or all of the flow has been pumped out for municipal or industrial use at least once and returned to the river.[1] This means that many of our sources of so-called fresh water are in effect partially diluted wastewater which has undergone varying degrees of treatment.

THE PERSISTANCE OF VIRUSES IN THE WATER ENVIRONMENT

The scientific and technical literature is replete with discussions of refractory chemicals not removed by treatment processes and non-biodegradable substances unaffected by the processes of natural self-purification of rivers. To this list must be added a group of enteroviruses which are amazingly resistant to environmental factors and resemble in many ways the behavior of the so-called refractory chemical wastes which persist so long.

Many laboratory and field studies have indicated that most enteroviruses can persist for days and many even for months in the natural water environment with viabilities longest in heavily contaminated water during cold weather.[2] Under most river conditions, where downstream consumers are rarely removed from upstream sources of fecal contamination by more than a day or so of flow time, it must be accepted that the enterovirus burden that reaches our water arteries is carried along essentially undisturbed. We have detected enteroviruses 25 km downstream of a single isolated source of sewage flow into the Jordan River,[3] and others have provided similar evidence of the resistant nature of the virus burden in the water environment and their persistance in rivers, lakes and in the sea even at points relatively far removed from the point of their introduction.

In 1962 an eminent panel of public health experts[4] concisely summarized their views of the enterovirus burden on water quality as follows:

> More than 70 viruses have been detected in human feces. All may be present in sewage. Viruses pass through sewage treatment plants, persist in contaminated waters, and may penetrate the water treatment plants. Numerous outbreaks of infectious hepatitis have been traced to contaminated drinking water. The occurrence of such incidents appears to be increasing.

Three years later another group of scientists concluded that "the capabilities of present water pollution control techonology are clearly inadequate as far as viruses are concerned."

TEN YEARS OF RESEARCH FINDINGS

In 1965 when Gerald Berg and his colleagues initiated the first international gathering of scientists to be devoted exclusively to the problem of viruses in water under the heading,"Symposium on the Transmission of Viruses by the Water Route," they asked: What is the relationship among viral diseases, water supplies and water pollution; what answers are needed to protect the public health from the transmission of viral diseases; what answers do we have; and what research is needed to obtain additional answers?

Ten years have passed since that first symposium and the handful of active pioneer researchers in the field of viruses in water has grown considerably and much progress has been made in helping to answer some of the questions that were then unanswered.

Virus monitoring techniques, although still not perfected, have been developed during this period to assay as much as 100 gallon samples of water, which was then but a far-off goal. Utilizing various monitoring methods, surveys of virus concentrations in the natural water environment have been conducted by researchers in many countries and all continents. We have a clearer picture today of the rate of enteroviruses shed by communities into the water environment. Raw wastewater often carries enterovirus loads of 100-1000 pfu per 100 ml.[3] Rivers which serve as receiving waters for raw sewage or treated effluent often carry between 1-10 pfu of enterovirus per 100 ml. Other studies have detected the presences of varying concentrations of viruses in lakes and sea water used for recreational purposes; in wells used to supply drinking water; in irrigation water drawn from polluted rivers; in sea water in areas of shellfish culture; and in the air in the vicinity of a wastewater spray irrigation project.

In fact, it appears that when well-trained scientists use any one of a number of new sensitive virus monitoring methods which can sample large enough volumes of water, they are able to detect enteroviruses in most phases of the natural water environment which is today almost universally exposed to sources of fecal contamination.

Here it must be added that so far, enteroviruses have been detected only rarely in drinking water supplies which have undergone conventional treatment. Of course, the chances of detecting viruses in finished water supplies have been slight, since for all intents and purposes there has been to date no program for routine virus monitoring of such water supplies except for the city of Paris, which has used the gauze pad method shown to have a relatively low efficiency.

During these past ten years many excellent studies have advanced our understanding of the virus removal efficiency of conventional as well as non-conventional water and wastewater treatment processes.

Here again, without going into details, it appears that although many advanced wastewater treatment processes such as lime precipitation, ozonization and others can remove a very high percentage of the viruses in wastewater, most normally designed and operated conventional biological wastewater treatment plants are capable of removing no more than 80-90 percent of the viruses and even when chlorination of effluent is practiced, it would be difficult to expect more than a 98.5 percent removal under most conditions.

The virus removal efficiency of most conventional water treatment plants is also not consistent although here too laboratory and pilot plant studies have demonstrated that much better removal efficiences can be obtained, particularly if break point chlorination or ozonization is practiced.

Studies indicate that where conventional high rate sand filtration plants followed by chlorination are treating heavily contaminated river water containing high concentrations of ammonia, it is unrealistic to rely on more than a 99.9 percent removal of enteroviruses from the stream.

THE LAG IN WATER TREATMENT PRACTICES

There is no doubt today that the statement made 10 years ago[5] that "the capabilities of water pollutional control technology is clearly inadequate as far as viruses are concerned," is no longer correct in light of new research findings and demonstration units. However, it remains essentially correct as far as actual practice in the field is concerned since most new plants designed and constructed during these past ten years have not taken into account upgraded standards of treatment that would be required to meet the virus problems which were so clearly defined in 1965.

Some have said that the risk of transmission of viruses by water, although being theoretically possible, is of no practical importance as a public health problem, but let us look briefly at the record.

TRANSMISSION OF VIRUS DISEASE BY WATER

Craun and McCabe[6] reported that in the United States there was a gradual increase from 1940 to 1970 in the number of cases per year of infectious hepatitis occurring in waterborne outbreaks while a reverse trend is noted for typhoid fever cases. The Center for Disease Control in Atlanta has reported that during the two year period of 1971-1972 there were 11 infectious hepatitis waterborne outbreaks involving 266 persons. These infectious hepatitis outbreaks represent the most prevalent type of waterborne outbreaks among those where the atiological agent was specified. It is true that these reported waterborne infectious hepatitis cases represent only about 0.3 percent of the total reported number of cases of infectious hepatitis. But one wonders how many secondary infections or even contact spread epidemics of the disease might have been initiated by a single clinical or subclinical case of infectious hepatitis introduced into a community as a result of ingesting a minimal infectious dose of the agent carried by the water supply. The massive outbreaks of infectious hepatitis transmitted by shellfish grown in sewage contaminated coastal waters underscore the potential public health risks associated with the growing burden of viruses in the water environment. Are we prepared to ignore this evidence even if it is not as definitive as one might prefer?

The conclusions of Plotkin presented at the 1965 Symposium[7] that ". . . one infective dose of tissue culture is sufficient to infect man. . ." still remains to this day the basis for Berg's[8] conclusion that "any amount of virus in drinking or recreational water that is detectable in appropriate cell cultures constitutes a hazard to those drinking the water." Nothing that has been reported in the literature since that time seems to contest that clear-cut conclusion.

THE CAUSE OF THE LAG

Why then has so little been done to translate the findings and conclusions of ten years ago, which have been further strengthened and reinforced by subsequent research, into public health policy and engineering reality? The knowhow is at hand and the technology has been tried and tested.

It seems that the first cause of hesitancy in translating the recommendations of the 1965 symposium into water pollution control policy was the lack of a clear-cut epidemiological basis for establishing a definitive virological standard for drinking and recreational water. The second and possibly more central reason was the absence of an acceptable standard procedure for assaying relatively large volumes of water for viruses. The availability of such a tried and proven method is the _sine qua non_ for establishing a virus standard for water. Let us see if these two reasons still hold today.

As to the first, the lack of a clear-cut epidemiological basis, let us look back and examine how the original microbial standard for drinking water was established in its day.

The fact that water can serve as a highly effective vector of enteric disease agents was reported by John Snow as early as 1854, even before there was a full understanding of the nature of the causative pathogenic microorganisms themselves. Methods of treating water by sand filtration to remove the causative agents were also practiced prior to the time that the efficiency of such processes could be controlled by evaluating the degree of removal of enteric microorganisms. However, as time went on, it became the practice to check the effectiveness of treating polluted water by bacterial testing.

In 1900 the slow sand filter at Lawrence, Mass., treating the highly polluted water of the Merrimac River, had been in operation for several years and its installation had been followed by a drop in the typhoid fever death rate in the city. Based on their slow sand filter performance and epidemiological insight they were able to set one of the first microbial standards for drinking water which in today's terms would be equivalent to 69 coliform bactera per 100 ml.[9] With the introduction of water disinfection technology by chlorine, it was possible in 1910 for the U.S. Public Health Service to establish its first statutory standard for drinking water at 2 coliform bacteria per 100 ml. of water. In both these cases the standard set was essentially a function of engineering feasibility based on the assumption that the less enteric organisms ingested in drinking water, the safer it would be.

Since that time, health authorities in many countries throughout the world have set similar numerical standards for the microbial quality of drinking water as one of the first administrative aids in the monitoring and control of the safety of water supplies. There can be little debate today as to the wisdom and foresightedness of those who established that early environmental quality standard which has made a major contribution in the prevention and control of waterborne enteric disease and protecting the health of hundreds of millions of people.

The bacterial standard for drinking water was established and put into practice long before there was a full scientific evaulation of such factors as: the minimal infectious dose; the ratio of pathogens to coliforms; the dose-response relationship; the reliability of coliforms as an indicator under various conditions in the natural environment; and the relative resistance of coliforms and pathogens in different water treatment processes.

In presenting this brief historical background of early microbial water standards, it is not to be implied that since the almost universally accepted coliform drinking water standard was established in its day, mainly on engineering feasibility criteria, rather than epidemiological and scientific data, that we should automatically do likewise today.

There is nevertheless something to be said for the basic logic and direct approach of the early public health pioneers. They had good reason to believe that by ingesting pathogenic bacteria, carried by water, people could and did become infected and they therefore strived within the limits of the technology available to reduce the potential risk to a reasonable minimum. They then set standards based on engineering feasibility so that others would have goals to meet which had been proved to be obtainable. These standards also enabled them to monitor and control water treatment plants once they had been built and put into operation so as to assure their continued high level of performance. In the case of coliform bacteria in drinking water where treatment costs were relatively small and benefits demonstratively large, the decision to set a microbial standard based on the lowest level obtainable under reasonable engineering conditions may in light of today's knowledge and experience by considered obvious and simple enough. However, 70 years ago there were many who opposed spending the money needed to build the water treatment plants required to meet those early standards. What may be obvious today was not so obvious to those who held the purse strings at the time.

Enough has been said and written elsewhere as to the inadequacies of the coliform standard in water as far as viruses are concerned. Most are in agreement today that the absence of coliforms in water--particularly water originating from a highly polluted source--is not under all circumstances an assurance that viruses are not present in low concentrations.

ANALYTICAL APPROACH

Lacking a clear-cut epidemiological basis for establishing a virus standard for water, let us attempt to arrive at one analytically. Let us assume that one tissue culture infectious dose may indeed lead to infection in a susceptible man and that one per hundred such persons exposed to such a dose will, in fact, become sick.

Let us also assume that the raw sewage stream contains 1000 pfu per 100 ml as had been demonstrated by a number of researchers in various parts of the world. Such a raw sewage stream might be treated by conventional methods leading to a 90 percent reduction of viruses and then diluted in a river by a factor of 100. We could expect a further reduction in virus concentration by about 90 percent due to sedimentation and natural die away in the stream so that at a downstream water treatment plant intake the virus concentration might now be 1 pfu per 1000 ml. If the water treatment plant is conventionally designed and operated, it should be capable of providing a further reduction in virus concentration of 99.9 percent. That would mean there still might be something like 1 pfu per 1000 liters in the community water supply. Or in other words, about one person per thousand might ingest a tissue culture infectious dose per day. If our assumption is correct that only 1 percent of those ingesting such a virus dose would actually become sick, this might mean one person per hundred thousand. Such a low level of infection might never be detectable in small communities and even in very large cities might not lead to the conclusion that the disease was waterborne, since most cases would appear as sporadic clinical cases of various virus diseases.

When one or a number of the virus inactivating barriers of such a system is inoperative and the virus concentration in the community water supply is increased by several orders of magnitude, there is the possibility that more easily detectable epidemic situations might develop, particularly if a virulent virus is being shed into the water from an upstream source. All of this is based on many assumptions but it is indicative of the type of situation that may exist today with current enterovirus burdens in our water environment and current water treatment technology.

THE NEED TO ESTABLISH VIRUS STANDARDS FOR WATER

It would not be unduly restrictive to draw the same conclusion as the early public health pioneers reached when they established the first coliform standard for drinking water based on engineering feasibility. It certainly should be technically feasible today to produce drinking water containing no more than 1 pfu per 1000 liters. Actually we know we can do better than that and we should insist on it wherever possible.

However, here we come to the second constraint--the limits of the virus monitoring methods. Despite the fact that we have not yet selected a single standard virus monitoring technique, a number of candidate methods capable of assaying at least 100 gallons of water have been developed and are reviewed in this report. Without too much additional effort it should be possible today to reach a tentative agreement on the use of one or more of the most reliable of the methods for routine water monitoring of 100 gallon samples.

The next step would be to set tentative virus standards for water. A number of proposals have been made. The World Health Organization has recommended[10] that drinking water should not have any viruses in a 10 liter sample. Melnick[11] has suggested that no more than 1 pfu per 100 gallons be allowed, while Berg[12] has stated the premise that the permissible level of viruses in water should be none.

Since it is obvious that any practical virus standard set today has to relate to both engineering feasibility and the capability of monitoring techniques to detect viruses in water, it would not be possible to set a standard calling for no viruses in water.

It is, however, feasible today to call for the establishment of a standard that we know can be met by well operated conventional plants and that we can verify by current monitoring techniques, even if we know that we should be able to do better and may well want to require a more rigorous standard later on as the technology develops, or for special cases such as wastewater reuse.

A standard which requires that no viruses be present in a 100 gallon sample of drinking water is a practical and attainable goal which we are capable of establishing and putting into affect right now. There are many reasons why such a standard might not be good enough, but we would make a lot of progress in upgrading water quality throughout the world if this modest standard was achieved universally. Far too many urban water supplies do not meet it today! To postpone setting a standard today in order to wait until we can establish a more refined one later will only result in a further continuation of the current lax attitude towards controlling viruses in water. A wise man once stated that the worst enemy of the "good" is the "best!"

VIRUS STANDARD FOR RECREATIONAL WATER

It might also be desirable to consider a virus standard for recreational water, although the epidemiological evidence is much less convincing. Studies have shown that bathers do actually ingest from 10-50 ml per bathing period and could swallow viruses present in the water. Melnick[11] has proposed that 1 pfu per 10 gallons be set as a standard for recreational water, while Cookson[13] has proposed a similar standard for effluent to be disposed into rivers used for recreational purposes. There is a particularly strong logic for a virus standard for seawater used for bathing since studies have shown that coliforms disappear exceedingly rapidly in the sea as compared to enteroviruses. We have shown that in the Mediterranean Coast of Israel, coliforms have a T_{90} of one hour or less while poliovirus has a T_{90} of one to two days. We have been able to detect enteroviruses at a bathing beach exposed to a known source of sewage although the coliform count at that beach was quite low.[3] A direct virus standard for recreational waters of no viruses in 10 gallons of water is feasible and should likewise be established today.

VIRUS STANDARD FOR RENOVATED WATER

The planned use of renovated wastewater for domestic consumption is a special case which would justify an even more rigorous virus standard due to the special risks that might be associated with such use.[1] Here virus monitoring techniques are not yet sensitive enough to serve as a basis for monitoring the virus removal efficiency of the whole treatment train since it would be reasonable here to insist on the lowest level of viruses that can be obtained by today's most advanced wastewater treatment technology. Berg[12] has suggested that renovated water be treated in such a manner as to destroy 12 log units of reference virus. A treatment train which includes chlorination with 10 mg/l of HOCl for one hour or an ozone residual of 0.5 mg/l for 15 minutes as reported on in the report, may well be capable of achieving this end. Here the virus removal capability of each step in the treatment train should be checked independently and the degree of safety of the processes be established in advance. In such an effluent, no enteroviruses should be detectable in a 100 gallon sample. When it becomes practical to monitor even larger samples, the standard could be upgraded accordingly. In order to provide the proper degree of safety to the public in cases where renovated water is being used directly for domestic supply, WHO has recommended that each batch be monitored for viruses before release to the distribution system. For this, a rapid method of testing for viruses in water is essential since conventional methods would require three days and more to obtain the results. The fluorescent antibody technique developed in our laboratories by Katzenelsun,[14] and reported upon here holds promise of providing an effective solution with quantitative virus assay results in 24 hours.

PRACTICAL PROBLEMS

In advocating that virus standards for water supplies and recreational water be established now a number of practical problems have to be overcome. Virus drinking water standard might be applied in the first instance only to cities having populations of 100,000 or greater which draw their raw water supplies from surface sources exposed to known fecal contamination. This would reduce the number of communities in the world which would have to carry out mandatory virus assays while concentrating on those that need it the most.

Although a few very large cities might find it feasible to establish their own virus laboratory to carry out all stages of the virus monitoring program it would not be practical or desirable for all communities to do this. Specialized national, regional or state laboratories could carry out the actual virus assay work for a number of communities. Under such a system the virus concentration procedure would be carried out by local personnel who would send the concentrated sample or filter cartridge to the regional laboratory for tissue culture assay. WHO has already recommended that each country establish national or regional laboratories capable of carrying out virus assays of water.[10] Many countries have such facilities or are in the process of organizing them.

There would be a need to establish one or several tentative standard procedures for concentrating and detecting viruses in water. There would also be a need to train additional laboratory personnel in virus concentration and detection techniques. The E.P.A. and the World Health Organization in cooperation with national health agencies should take the lead in establishing the tentative standards and training personnel in their application.

In a matter of a few years' time it might well be possible to put the proposed virus standards for water into practice, at least in a number of the more highly developed countries where such a requirement could best be justified. Although trained personnel and laboratory facilities may be a limiting factor at first, it should not take too long to overcome.

CONCLUSIONS

A built-in paradox of modern society revolves around a situation where more and more water is needed by the growing population but less and less becomes available at the required quality as a result of the self-destructive process of pollution. The increasing burden of highly persistent enteroviruses in the water environment on water quality is of concern not only in relation to drinking water, but in water used for recreation, agricultural irrigation and growing shellfish.

Enteroviruses have been detected in all phases of the water environment and conventional water and wastewater technology is not capable of removing such viruses under all circumstances in an entirely reliable manner. Although only a very small percentage of the total number of reported infectious hepatitis cases per year is associated with waterborned outbreaks, the number of infectious hepatitis cases per year associated with such outbreaks is on the rise and is today the single most prevalent type of waterborne disease of known etiology. Massive epidemics of infectious hepatitis transmitted by shellfish grown in sewage contaminated water underscore the potential risks associated with the growing burden of viruses in the water environment.

It is justifiable today to establish a virus standard for water on the basis of technological feasibility. It should be possible even with conventional technology to produce a drinking water with less than 1 pfu per 1000 liters. It is also possible today to monitor 100 gallons of water for the presence of viruses. A tentative standard of no viruses in 100 gallons of water would be a good first step in upgrading water treatment facilities throughout the world. Virus standards should be set for recreational water and renovated water as well. We have waited too long for research to provide the "perfect" virus monitoring technique and the most efficient method for removing viruses from water. In the interim little has been done in practice to apply what we already know about the need to control viruses in water. Let us not wait for the "best" but try to apply today what can be considered "good enough." The establishment of a virus standard in water will be a real step forward in achieving much needed improvements in water quality.

RESEARCH PROGRAM

The sections that follow in this report review studies carried out at the Hebrew University of Jerusalem over a five year period in the development and evolution of virus monitoring and detection techniques and in the inactivation of enteroviruses in water with ozone. These studies have furthered the basic understanding of the problems of viruses in water and their control and should provide a basis for both practical applications and further investigations.

REFERENCES

1. World Health Organization. Reuse of Effluents: Methods of Wastewater Treatment and Health Safeguards. Geneva, Technical Report Series No. 517, 1973. 63 p.

2. Akin, E.W., W.H. Benton, and W.F. Hill, Jr. Enteric Viruses in Ground and Surface Waters: A Review of Their Occurrence and Survival. In: Viruses and Water Quality: Occurrence and Control, Snoeyink, V., (ed.). Urbana, Ill., University of Illinois, 1971.

3. Shuval, H.I. Detection and Control of Enteroviruses in the Water Environment. In: Developments in Water Quality Research, Shuval, H.I., (ed.). Ann Arbor, Mich., Ann Arbor-Humphrey Science, 1970.

4. U.S. Department of Health, Education and Welfare. Report of the Committee on Environmental Health Problems to the Surgeon General. U.S. Government Printing Office, Washington, D.C. USPHS Publ. No. 908. 1962.

5. President's Science Advisory Committee. Restoring the Quality of Our Environment. Report of the Environmental Pollution Panel, Washington, D.C. 1965. 317 p.

6. Craun, G.F., and L.J. McCabe. Review of Causes of Waterborne Disease Outbreaks. J. Amer. Water Works Ass. 65:74-84, 1973.

7. Plotkin, S.A., and M. Katz. Minimal Infective Doses of Viruses for Man by the Oral Route. In: Transmission of Viruses by the Water Route, Berg, G. (ed.). New York, John Wiley and Sons, 1967.

8. Berg, G. (ed.). Transmission of Viruses by the Water Route. New York, John Wiley and Sons, 1967. 484 pp.

9. Phelps, E.B. Public Health Engineering. New York, John Wiley and Sons, 1948.

10. World Health Organization. International Standards for Drinking Water. 3rd ed. Geneva, World Health Organization, 1972. 70 p.

11. Melnick, J.L. Detection of Virus Spread by the Water Route. In: Viruses and Water Quality: Occurrence and Control, Snoeyink, V., (ed.). Urbana, Ill., University of Illinois, 1971.

12. Berg G. Integrated Apporach to Problem of Viruses in Water. J. San. Eng. Div., Proc. Amer. Soc. Civil Engrs. 97: SA6:867-882, 1971.

13. Cookson, J.T. Report on the Use of Temporary Wastewater Treatment Plants: Standards and Procedures for the Elimination of Health Hazards. Maryland, Montgomery County Council, 1972.

14. Katzenelson, E., Virologic and Engineering Problems in Monitoring Viruses in Water. In: Viruses and Water, Berg, G., H.L. Bodily, E.H. Lennete, J.L. Melnick and T.G. Metcalf, (eds.). Washington, D.C., American Public Health Association, 1975.

SECTION II

CONCLUSIONS

1. A methodology was developed to test the effectiveness of different systems for concentrating water samples for the isolation and detection of viruses using standardized conditions so as to obtain comparable results. The following eight different methods for virus concentration and detection were studied for sample concentration ability and virus recovery efficiency, using mainly 5 liter samples:

    1. Aluminum alginate ultrafiltration (according to Gartner).[1]
    2. Cellulose nitrate membrane filtration (according to Berg et al).[2]
    3. Aluminum hydroxide precipitation (according to Wallis and Melnick).[3]
    4. Phase separation (according to Shuval et al).[4]
    5. Flow-through gauze sampler.
    6. Cellulose nitrate membrane filter (according to Wallis et al).[5]
    7. Insoluble polyelectrolytes (PE 60) (according to Wallis and Melnick).[6]
    8. Cellulose nitrate membrane filter-eluting method (according to Rao and Labzoffsky).[7]

2. The methods which showed consistently very high recovery efficiencies with poliovirus of about 100% were aluminum alginate ultrafiltration, aluminum hydroxide precipitation and phase separation.

3. With seeded Echovirus 7 the Alginate and Phase Separation methods showed about 100% virus recovery efficiency while the other methods studied showed lower recoveries.

4. When natural wild types of enteroviruses from sewage were seeded in water to be tested by various concentration methods, the efficiency of virus recovery was generally lower except for the membrane filtration method (according to Rao et al).[7]

5. The tests for recovery efficiency with natural wild strains of enterovirus indicate that a number of the methods may be selective in concentrating enteroviruses which would mitigate against their use for field monitoring purposes. Three methods, membrane filtration (according to Rao et al),[7] membrane filtration (according to Wallis et al)[3] and PE 60 (according to Wallis and Melnick),[6] showed reasonably good levels of virus recovery of 65% and more with both poliovirus alone and with natural wild strains of enteric viruses.

6. An in-depth study of the gauze pad method made it possible to provide a quantitative evaluation of this much-used, simple technique. The efficiency of virus recovery for large volumes of water passed through the gauze pad was under 1%.

7. Recovery efficiency of over 100% was found in many experiments and may be explained by the presence of virus clumps in the samples

which may become disaggregated during the concentration procedure. Experimental evidence cited from other studies supports this possibility.

8. Of the above eight methods studied the alginate and phase separation methods are limited for practical purposes to concentrating samples of 10 liters or less and are therefore not applicable to monitoring drinking water samples which should have a volume of at least 100 gallons. The cellulose nitrate membrane filtration, aluminum hydroxide and PE 60 methods are suitable for such large volumes of water and demonstrate reasonable recovery efficiencies.

9. The phase separation, PE 60 and gauze pad method can be used directly for samples of sewage and highly contaminated water while the other methods are effective only with water samples relatively free from suspended solids unless special arrangements for prefiltration are provided.

10. A new, promising virus concentration technique using cellulose acetate hollow fiber ultrafiltration membranes was developed and evaluated. Large volumes of water can be concentrated rapidly by this method with reasonable virus recovery efficiency. The method has the advantage of not requiring pH adjustment of the feed water as required with the cellulose nitrate membrane filtration method.

This method is not adversely affected by the presence of organic contaminants in the water. It may prove to be an effective first stage in a sample concentration train to be followed by other sample concentration techniques.

11. Attempts at a quantitative evaluation of the efficiency of the gauze pad method under laboratory conditions, indicate an inverse relationship between efficiency of recovery and sample volume. At low volumes of 700 ml, the efficiency of recovery was 7% while with 50 liters only 0.5% virus recovery was achieved.

12. In field studies using the gauze pad, it was demonstrated to be more effective than 2 liter grab samples taken from the same sewage stream. The concentration of viruses in the liquid expressed from gauze pads immersed in a sewage stream was about 100 times greater than that found in unconcentrated grab samples taken from the sewage in which it was immersed. No advantage was found in keeping the pad immersed longer than 24 hours in the water stream being tested. Despite its low-efficiency of recovery and non-quantitative nature, the gauze pad can be of some value in field survey when other more refined sample concentrating techniques for large volumes are not available.

13. A rapid fluorescent antibody method for quantitative isolation of viruses from water has been developed using poliovirus type I as model. This method has been shown to be capable of giving a qualitative answer as to the presence of viruses in a concentrated

water sample in as little as 6-9 hours and a quantitative estimate of the virus concentration in 18 hours. The FA quantitative assay has been shown to be comparable to that obtained by the conventional plaque forming method which requires about 3-7 days to complete.

14. A study of virus types in sewage in Israel indicates that of 489 strains isolated and identified, 74% proved to be poliovirus. Ten percent were coxsackie type B or Echo 9 while 16% were other Echovirus types or other viruses. Thirteen percent of the poliovirus strains showed strong CPE when incubated at 40°C which is generally considered to indicate wild pathogenic strains as compared to the remaining 87% which grew only at 37°C. These latter isolates are considered to be attenuated poliovirus vaccine strains. Further field studies, using both grab samples and gauze pads checked for poliovirus by neutralization tests, also indicate that poliovirus is usually present. Poliovirus is ubiquitous in Israel sewage and can be detected in essentially every sample tested. This is to be expected in a country practicing routine immunization of all infants against polio with live vaccine. These findings may provide some support for the possible use of poliovirus as a virus indicator organism using the rapid FA method.

15. The chemistry of ozone as a disinfectant is reviewed with possible explanations for the chemical pathways leading to the formation of its active radicals. The possibility that a dissociation product, hydroxylradical (OH), rather than ozone itself is the active disinfectant is also presented. Evidence is presented to show that the

disinfectant power of ozone is pH dependent, increasing as pH rises. This may provide some support for the hypothesis that the OH radical is the active germicidal species.

16. A spectrophotometric method for the determination of ozone in small samples of water was developed and evaluated. This method has been shown to detect ozone concentrations as low as 0.01 mg/l in a 10 ml sample.

17. Studies on the stability of ozone in aqueous solutions indicate at 5^{0}C ozone concentrations show only minor decreases in 30 minutes, while at 22^{0}C the concentration was reduced by about 50% in 30 minutes. Ozone solutions stirred at 100 rpm resulted in a rapid loss of ozone while at 80 rpm the ozone concentration was more or less stable over 30 minutes. Studies also showed that unless very carefully purified, virus stocks caused rapid loss of ozone. The results of these studies assisted in establishing conditions for virus inactivation kinetic studies.

18. Ozone has been found to have a very rapid virucidal effect in a low ozone demand system with constant ozone levels. For example, 99% of inoculated poliovirus is inactivated in less than 10 seconds with an ozone concentration of 0.3 ppm as compared to 100 seconds required for the same degree of kill with 0.3 ppm of chlorine or 100 minutes for the same concentration of iodine

19. The inactivation kinetics curve shows two stages. The first rapid kill stage lasting less than 10 seconds with inactivations usually

of 99% and more. The second stage shows a lower rate of kill with essentially total inactivation of all virus present being completed in several minutes.

20. Residual ozone concentrations under 0.1 ppm produce irregular rates of virus inactivation with no inactivation found in many tests, while increasing ozone concentration above 0.2 ppm to 1 ppm had very little effect on the virus inactivation rate. This finding might be interpreted to support the "all or nothing" effect attributed to ozone.

21. Some of the irregular and unpredictable inactivation patterns of ozone on viruses resulted from changes in the degree of resistance to ozone of certain virus stock cultures. It has been shown that virus stock culture highly resistant to ozone results from transferring virus stock from -70°C to -18°C. Clumping may be the responsible mode in these resistant virus cultures. It is not clear whether wild virus strains in nature are of the resistant form or not.

22. Ozone inactivation of Coliphage T_2 and E. coli followed similar patterns as that found with viruses but some indication of dose response patterns could be detected.

23. It is hypothesized that because of its extremely rapid virucidal action it was not possible to detect normal dose-response expected in any normal chemical disinfection reaction. Preliminary tests of virus kill in very short reaction periods were made and provide the first tentative evidence of a true dose-response relationship.

24. The virus inactivation ability of ozone was tested in clarified diluted sewage having B.O.D. levels of 50 and 25 ppm. In batch tests initial ozone doses of up to 1.0 mg/l were rapidly dissipated. However, with initial ozone doses of 0.8 mg/l, a 90% inactivation was achieved in 10 seconds with the more concentrated sewage and a 99.5% inactivation was achieved with the more dilute sewage, while in neither case was an ozone residual detectible. Organic matter concentrations in the substrate were shown to seriously interfere with the inactivation process.

25. In experiments with clarified raw sewage (B.O.D. = 500 ppm) and continuous bubbling of ozone, virus inactivation occurred before ozone residuals could be detected. A 99.9% inactivation of virus was achieved with an ozone residual of 0.6 mg/l. Virus inactivation levels greater than 99.9% were achieved with ozone residuals of 1 ppm in a matter of minutes. These data indicate that ozone is a very rapid and effective virucidal agent even in wastewater high in organic content.

26. Studies on natural inactivation of viruses in the marine environment show that specific marine bacteria possess antiviral activity. The presence of organic matter in the seawater inhibits the antiviral effect. The mechanism of this antiviral effect has not been determined.

REFERENCES

1. Gartner, H. Retention and Recovery of Polioviruses on a Soluble Ultrafilter. In: Transmission of Viruses by the Water Route. Berg, G. (ed.). New York, Wiley and Sons, 1967. p. 121-127.

2. Berg, G., D.R. Dahling, and D. Berman. Recovery of Small Quantities of Viruses from Clean Waters on Cellulose Nitrate Membrane Filters. Appl. Microbiol. 22:608-614, 1967.

3. Wallis, C., and J.L. Melnick. Concentration of Viruses on Aluminum Phosphate and Aluminum Hydroxide Precipitates. In: Transmission of Viruses by the Water Routu, Berg, G. (ed.). New York, Wiley and Sons, 1967. p. 129-138.

4. Shuval, H.I., B. Fattal, S. Cymbalista and N. Goldblum. The Phase Separation Method for the Concentration and Detection of Viruses in Water. Water Res. 3:225-240, 1969.

5. Wallis, C., M. Henderson, and J.L. Melnick. Enterovirus Concentration on Cellulose Membranes. Appl. Microbiol. 23:476-480, 1972.

6. Wallis, C., and J.L. Melnick. Detection of Viruses in Large Volumes of Natural Waters by Concentration on Insoluble Polyelectrolytes. Water Res. 4:787-796, 1970.

7. Rao, N.V., and N.A. Labzoffsky. A Simple Method for the Detection of Low Concentrations of Viruses in Large Volumes of Water by the Membrane Filter Technique. Canadian Jour. of Microbiol. 15:399-403, 1969.

SECTION III

RECOMMENDATIONS

1. While the comparative virus concentration and detection studies reported upon here were carried out with 5 liter samples, the goal for virus monitoring of water and wastewater should be the assay of large volumes of water--400 liters and more. A number of the methods evaluation are suitable for sampling such volumes, particularly filtration methods using cellulose nitrate membranes, aluminum hydroxide, PE 60 and the hollow fiber membrane technique. Further work in developing and evaluating these methods under controlled conditions with large volumes of water is called for.

2. Further study is required to determine the effect on virus recovery efficiency of organic matter, turbidity and variation in dissolved mineral content of the water samples since preliminary data indicates that these factors can be of major importance.

3. Further studies should be carried out to determine which of the methods shows the least selectivity in concentrating viruses from water samples. In the studies reported upon here, membrane filtration methods and PE 60 showed the least selectivity with natural wild strains of enteric viruses.

4. Further studies are required to determine the factors involved in virus recovery efficiencies which are apparently greater than 100% of the virus input. The preliminary findings that this phenomenon may be associated with virus clumping and disaggregation should be fully investigated.

5. Concentration of viruses from large volumes of water by filtration and elution will invariably require a second step to reconcentrate the elute to a volume small enough to be assayed conveniently in tissue culture cell systems. Since the efficiency of the second step is just as critical as the efficiency of recovery of the first step, it is essential that ways be found to maintain high recovery efficiency in it as well. This question requires further investigation.

6. The guaze pad method, while shown to have a low virus recovery efficiency of under 1% can be a useful nonquantitative field survey technique when suspended in a flowing stream for a 24-hour period, particularly when more sophisticated methods are not available. Such a sampling technique is in most cases more effective in detecting viruses in the stream than small grab samples.

7. The filtration methods which proved to be most efficient and which are applicable to testing large volumes of water require some

form of pretreatment when water high in suspended solids must be assayed. Appropriate prefiltration methods must be developed and tested to determine the extent that viruses attached to suspended particulates are removed by the pretreatment step.

8. The hollow fiber membrane concentration technique developed and tested during this study holds particular promise since it has a high virus recovery efficiency and can be designed to handle large volumes of water. In addition it does not require pH adjustment of the stream for virus adsorption nor is it adversely affected by the presence of organic matter and suspended solids in the water. The hollow fiber membrane system may prove to be an effective first stage in a sample concentration system followed by other concentration techniques. Further intensive study should be devoted to upscaling the system large volumes of 400 liters and more and testing its efficiency under field conditions.

9. The flourescent antibody (FA) method for the rapid quantitative assay of poliovirus in water should be of particular value where rapid results are required. A study as to the feasibility of using this method based on the concept of using poliovirus as an "indicator organism" of virus contamination in water should be field tested in various communities where routine polio vaccination is practiced and

it can be assumed that poliovirus is continuously present in the sewage system.

The possibility of expanding the technique to other enteric viruses should be studied.

10. Various tissue culture cell lines should be evaluated and compared to primary tissue culture cells to determine the most effective system or combination of systems for assaying natural wild virus strains from field samples.

11. Further study of the chemistry of ozone and ozone species in aqueous solutions and their disinfection ability is essential to gain a better understanding of the use of ozone against viruses under various conditions. The present state of lack of predictability is a major drawback in the use of ozone with natural raw waters or wastewater rich in organic compounds or other factors that may react with ozone.

12. The effect of pH should be further studied to determine whether the increased effectiveness of ozone with high pH is a result of more active ozone species or some change in the virus which might increase its sensitivity to ozone.

The role of clumping and disaggregation of viruses at various pH levels must be investigated in relation to this question.

13. Techniques must be developed to study the kinetics of ozone inactivation at very short time periods, ranging from one-half to 10 seconds. Preliminary evidence presented in this study indicates that while there is little or no detectable dose response relationship for contact times greater than 8 seconds there may be such a relationship at shorter times. Such studies are essential to an understanding of this question.

14. Findings relating to the shifting of ozone sensitive virus cultures to ozone resistant ones as a result of transferring stock cultures from -80^0C to -18^0C points to the need to assure rigorous standardization of virus inactivation evaluation procedures. Further investigation should be made as to the mechanism associated with this phenomenon particularly to determine the role of clumping.

Studies should also be made to determine whether viruses in nature, i.e., in sewage, are of the sensitive or resistant form.

15. Ozone has been shown to be a very rapid and effective virucidal agent in clean water as well as in wastewater effluent and holds promise of being of great practical value in field application particularly where it is not considered desirable to use ever-increasing chlorine doses. Further studies on the use of ozone as a virucidal agent under field conditions with various types of water are required.

16. There is a need to investigate the mechanism of virus kill by ozone so as to allow for more rational approach to its use.

17. In cases where a residual disinfectant is considered desirable in the water supply system, combinations of ozone and chlorine can be considered. The reactions between ozone and chlorine both in clean and raw water must therefore be studied.

18. If ozone is to be used instead of chlorine both because of its greater virucidal power and due to concern over the formation of toxic organohalides resulting from chlorination, there is a need to determine whether the ozonization of organics in water results in the production of any deleterious compounds. Such studies are an essential phase in the full evaluation of ozone disinfection for mass field application.

PART A

VIRUS DETECTION METHODS

SECTION IV

A REVIEW OF METHODS FOR THE DETECTION OF ENTERIC VIRUSES IN THE WATER ENVIRONMENT

INTRODUCTION

The possibility that water might serve as the vehicle for the transmission of certain virus diseases, particularly those whose infectious agent is excreted through the enteric tract, has been considered feasible for some time. Mosely[1] has pointed out that over 50 documented water-borne epidemics of infectious hepatitis have been recorded over the years. Apart from infectious hepatitis, poliomyelitis and viral gastroenteritis were the only other viral infections that caused epidemics suspected of being transmitted by water. In most cases, the epidemiological evidence was inconclusive. An exception is possibly a small polio epidemic in Nebraska in 1952[2] where strong evidence suggested that it was caused at least partially by a water-borne virus. The possibility that viral gastroenteritis may be water-borne on occasion cannot be ruled out, however.

The massive waterborne epidemic of infectious hepatitis in Delhi, India, in 1955[3] in which some 30,000 persons became infected by the contaminated municipal drinking water which had undergone what is generally considered complete and adequate treatment, including chlorination, emphasized the need to develop new methods of monitoring water supplies for viruses.

Over the years, evidence has pointed to the fact that the usual bacterial parameters of water purity, particularly the coliform group, may not provide an adequate index as to the safety of water from a virological point of view. The need to monitor water specifically for viruses of enteric origin presents many problems, and the developments in this field will be reported here.

THE PRESENCE OF ENTERIC VIRUSES IN WATER

Over 100 virus types are known to be excreted from humans through the enteric tract and may find their way together with sewage into sources of drinking water. Many of these viruses are known to cause disease in man. However, the critical question is whether these viruses can survive long enough and in high enough concentration to cause disease in people consuming such contaminated water. The concentration of enteric viruses in sewage and polluted water is an important factor to consider. Clarke and Kabler[4] calculated a theoretical average number of enteric virus in infectious units in sewage and found it to be about 500/100 ml. In our own studies we have found the enteric virus concentration in the sewage of communities in Israel range from 10-100/ml.[5] Based on these figures it can be assumed that the virus concentration in polluted river water would range from 1-10 viral infectious units/100 ml as a result of physical dilution only. The number is lower during the cold months and somewhat higher in the late summer and early fall due to seasonal variation of enteric virus diseases. It can be assumed that this concentration will be further reduced both by processes of natural die-away and by water treatment, imperfect as they may be in the removal and inactivation of viruses.

Under normal circumstances only a relatively small number of infective units will, at worst, penetrate a water supply system which derives its raw water from a heavily contaminated river. Simultaneous infection of a large number of people is therefore rather improbable under normal conditions with modern water treatment methods. Sporadic infections, however, are possible, at least theoretically. The latter becomes true particularly in the light of Plotkin and Katz's[6] claim that "one infective dose of tissue culture is sufficient to infect men if it is placed in contact with susceptible cells." They were able to reach this conclusion as a result of studies on attenuated polio viruses, respiratory viruses, agents of ocular diseases, viruses of animals, and other agents. This might mean that even when virus concentrations as low as one virus infectious unit per 1000 ml are present in water, a certain number of individuals might well become infected by consuming the normal daily intake of 1-2 liters per capita.

With this in mind, one may form a picture of water as playing a small but important role in the spread of viral diseases in man in areas provided with modern treatment facilities. The effect of such slightly contaminated water may lead to sporadic cases of disease dispersed over a large area. However, these occasional cases may, in turn, act as foci and through food or personal contact cause epidemics which may involve much larger numbers of people. In other cases when heavily contaminated water reaches large population groups without adequate treatment, explosive mass epidemics have occurred and may well occur in the future. It is therefore obvious that the development of methods for the detection of viruses in water are required to allow for an adequate evaluation of the virological safety of water supplies and treatment processes. Bacterial evaluation of water as an indicator of contamination cannot replace such methods since it became apparent that viruses are not as sensitive as bacteria to hostile environmental factors or to standard purification procedures, and they may be present in water even when bacterial counts are at acceptable standards.[7,8]

TYPES OF VIRUSES IN WATER

Water being used for drinking and bathing can act as a vehicle for the transmission of most viruses. The picorna group of viruses is the one most commonly found in sewage; it includes the polio, coxsackie, and echo viruses. Adeno viruses which cause respiratory and eye infections, and sometimes diarrhea, are commonly found in feces.

Infectious hepatitis is actually the only disease for which a water-borne infection has been proven beyond any doubt. However, its viral characteristics are not yet clear. There are some claims that the responsible virus has been isolated from suspected cases of hepatitis, but most virologists feel that these claims are as yet insufficiently established. It must be remembered, however, that in spite of the latter there is strong evidence supporting the view that this disease is actually caused by a virus.[9]

ISOLATION AND IDENTIFICATION OF ENTERIC VIRUSES

Viruses can only multiply inside living cells and therefore live organisms, such as animals, chick embryos, or tissue culture must be used for their isolation in the laboratory. For the enteric viruses, tissue cultures which may be of two types are generally used: primary tissue cultures and continuous cell cultures. Primary tissue cultures are usually prepared according to the method of Enders et al.[10] This method is based on the fact that 0.25% trypsin acts on small cuts prepared from a tissue (usually a kidney) by separating the cells from each other. When put inside a suitable glass or plastic flask, tube, or plate together with a tissue culture nutrient medium, these cells attach to the wall of the vessel and multiply. As a result, a monolayer of cells is formed on the wall. Continuous culturus are very similar, but instead of a tissue from an organ, a tissue culture is used as a source for the cells. Initial isolation of enteric viruses is usually done on primary tissue cultures prepared from monkey kidneys which typically have a higher sensitivity than most cell lines. However, any isolated virus can be adapted to cultures of the continuous type.

After inoculation of a virus into a tissue culture some of the cells become infected. The virus multiplies within these cells and spreads to the neighboring cells. At the same time, the infected cell usually undergoes morphological and biochemical changes and dies. The result is a slow process of destruction of cells in the culture, a phenomenon known as the cytopathic effect (CPE). The process of viral spread from cell to cell can be slowed down by adding a layer of agar together with tissue culture medium over the cells. As a result, instead of being rapid and confluent, the CPE will be limited to a smaller area which looks macroscopically like a hole in the monolayer of cells. These holes are also known as "plaques". A single plaque usually originates from a single infected cell which may be caused by a single virus infectious unit. This method is used for quantitation of enteric viruses in tissue cultures in the same manner as agar plates are used in bacteriology for the determination of bacterial counts. The term "plaque forming unit" (pfu) was given to the lowest concentration of viruses that form one plaque on a monolayer of cells.

Different viruses cause cytopathic effects which differ morphologically. Also, plaques may be of different sizes and shapes. However, this phenomenon cannot be used for the final identification of the isolates since some viruses, belonging to different groups, cause identical CPE.

Final identification can only be achieved with specific antisera. Here the identification is based on the fact that specific antiserum will neutralize the effect of the virus against which it was prepared.

QUANTITATION OF ENTERIC VIRUSES

Two methods are available for the quantitative determination of enteric viruses in a given sample of material being assayed, both of which give accurate results. Selection of the method to be used is usually based on the experience and resources of the laboratory. In the first one, the tube assay method, serial dilutions of the virus suspension to be tested are prepared. Groups of tissue culture tubes are inoculated with each dilution. After proper incubation at 37°C, the inoculated tubes are examined for CPE. Quantitation is obtained by finding the lowest dilution of the virus suspension that caused CPE in 50% of the tubes. The figure obtained is known as the $TCID_{50}$ (tissue culture infectious dose-50%) value of the virus suspension. Using this same method, it also is possible to calculate the virus concentration as a most probable number (MPN).

In the second, the plaque assay method, quantities of 0.3-1.0 ml of virus dilutions are inoculated into plates or bottles, the cells of which are later covered with an agar overlay. After proper incubation, usually at 37°C in a humid atmosphere containing 5% CO_2, the inoculated tissue cultures are examined for the presence of plaques. When plaques are present, they are counted and their number for each of the dilutions is determined. The number of pfu in the original virus suspension is then calculated and the virus concentration is reported as pfu/ml or other unit of volume.

A large number of methods for the isolation of virus from water have been developed over the years. A common step in almost all these methods is the concentration of the virus from the water sample. This is an integral part of the procedure since the quantity of viruses in water is often very small. Raw domestic sewage, the main source of viral contamination in water, contains about 10,000 plaque forming units (pfu) per liter. This number, however, may sometimes increase. In our laboratory we recently found up to 10^5 pfu/liter raw domestic sewage. When the virus number reaches such high proportions their isolation becomes relatively simple; but already at a level of 10^3 pfu/liter concentration of the virus is advisable if an exact quantitative evaluation is to be achieved. At lower virus levels concentration becomes a necessity. The basic difficulty in the isolation procedure is the fact that usually the inoculum of a tissue culture has a volume of 0.1-1.0 ml. In other words, when virus concentration in water is less than one per milliliter, it will require many inoculations. It is much simpler to concentrate the viruses from large volumes of fluid and to use this concentrate as the inoculum. Most systems for the isolation of viruses from water are therefore primarily concentration methods. In fact, the different concentration methods can be divided into seven main groups.

1. Sample Incorporation

In this method the conventional culture medium is so concentrated as to allow for the incorporation in it of 10-60 ml of the sample to be assayed.[11] According to another approach, large volumes of a maintenance medium, prepared from the water that is being tested, are inoculated onto cell cultures.[12] No concentration of virus from the water prior to inoculation is attempted in these cases. Nevertheless, by this method virus detection is enhanced since with the same tissue culture tube or bottle a volume of sample 10-20 times larger than normally inoculated can be assayed.

2. Ultrafiltration

The sample is passed through a filter with a pore size small enough to hold back the virus, which remains either on or in the filter, as is the case with the aluminum alginate filter.[13] After filtration, the filter is dissolved in a small volume of 3.8% sodium citrate solution providing a virus concentrate.

A more sophisticated method of ultrafiltration is to let the water flow over the surface of the filter thus preventing the virus and suspended solids from entering the filter and clogging it. The movement of the water is caused by constant stirring or by tangential direction of the flow.[14] The end volume, which has not passed through the filter is small and contains the concentrated viruses. Cellulose acetate membranes are used in this system mainly.

Hydroextraction,[15, 16] or its more advanced form, osmotic filtration,[17] also belong in the ultrafiltration group. Here, water is extracted from the sample through a dialysis membrane, either by hygroscopic material or by a concentrated salt solution.

3. Freezing

The sample is slowly cooled to -15°C under constant stirring. The ice crystals formed during the freezing process are pushed toward the periphery. Ultimately, a small volume of super cold water remains in the center containing all the substances that were present in the original sample: salts, colloids, suspended solids including viruses.[18]

4. Two-phase separation

This method is based on the discovery of Albertsson[19] that the result of certain mixtures made from two polymers such as dextran sulfate and polyethylene glycol in an aqueous solution leads to the formation of a two-phase system. Introduction of particles and macromolecules into this system will result in the partition of the particles in the two phases, depending on their size and surface properties. Viruses show a nearly one-sided distribution and the method may therefore be used for their concentration.[20,21] The concentration is accomplished by adding polymer solutions to a virus suspension in such proportions that almost all the virus particles are collected in a small volume bottom phase and may be drained off separately.

5. Ultracentrifugation

The centrifuge is mostly used for the concentration of small suspended particles from fluids. Being extremely small particles in the size range 20-200mμ, viruses are no exception, but because of their small size, relatively high forces of the order of 60,000 x g for one hour are required. These are obtained by using an ultracentrifuge. In the usual procedure, the water sample is first centrifuged at a relatively low speed to reduce the number of larger particles including bacteria. The supernatant is then centrifuged at high speed. The sediment obtained contains the virus and is [illegible]ended in a small volume of tissue culture medium. A high concentration factor may thus be reached. Anderson et al[22] described the development of a complex centrifugation system for the isolation and separation of small numbers of virus particles from large volumes of fluid.

6. Electrophoresis

Viruses are usually negatively charged at neutral pH values[23] and will therefore move toward the cathode when a virus suspension is placed in an electric field. This principle was used by Bier et al for concentrating bacteriophages in water.[24] A simple procedure was developed by which electrophoretic transport was used to bring about adsorption of bacteriophages on dialyzing membranes. With this method a sample of water could be processed in a relatively short time and a concentration of viruses achieved. Forced flow electrophoresis[25] and electro-osmosis[26] are two more advanced methods based on the same principle.

7. Adsorption and Elution

With this method viruses are adsorbed from water onto either cotton,[27] aluminum hydroxide,[28] protamine sulfate,[29] insoluble polyelectrolytes,[30] or cellulose nitrate,[31] which are amongst the adsorbants most often used. The adsorbant is sometimes added to the water sample in powder form and separated by centrifugation or simple filtration. A second approach is to pass the sample through a layer of adsorbant placed directly on a filter, or the adsorbant itself fulfils the role of filter. The viruses are usually eluted from the adsorbant by a small volume of either alkaline[32] or a protein-rich[31] solution.

The large variety of methods is impressive and is still increasing, which underscores the importance of viruses in water. But, at the same time, it also indicates that the methods are still lacking in proficiency.

What do we expect from the ultimate virus isolation method? It should be able to quantitatively isolate in a very short time all the different viruses found in large volumes of various qualities of water. Each of these requirements present their own specific problems, and we shall therefore discuss them one by one.

VIRUS TYPES AND RECOVERY EFFICIENCY

More than a hundred different virus types are found in domestic sewage. Most of them belong to the picorna group, which include poliomyelitis, coxsackie and echoviruses. But reo- and adeno viruses are also often isolated from sewage. This great divergency in virus types poses the first difficulty--the lack of one single system for their isolation. For example, many of the coxsackie viruses multiply in suckling mice, but not in tissue cultures. Echo and polioviruses, on the other hand, multiply in tissue cultures only. This is an extreme case. Generally, different virus types favor specific tissue cultures.

Two virus types found simultaneously in the sample, may prefer the same kind of tissue culture, but may differ in their multiplication rates as is seen with polio- and reoviruses. The poliovirus proliferates at a greater rate than the reovirus and thus destroys the cell culture while the reovirus is still in its initial stages of development. This phenomenon is especially noticeable when using the plaque-count method. On the other hand, the tissue culture infective dose ($TCID_{50}$) method allows the more slowly multiplying virus to appear. Thus Hupen[33] found the $TCID_{50}$ method for detecting and quatitation of viruses in sewage superior to the pfu method.

In addition to the isolation system, the concentration method, too, may be selective. Grindrod and Cliver[34] found the two-phase technique a significant aid in the detection of all poliovirus types, coxsackie virus type B-3, and coxsackie virus type A-9. The method was found to be ineffective for samples containing coxsackie virus type B-2 and echo virus type 6. The lower phase, into which the viruses concentrate, is strongly inhibitory to these two agents and to influenza A(PR-8) virus, suggesting that the effect might extend to other viruses as well. They therefore warn users of this procedure in environmental virology studies to be aware of the fact that their results may be biased significantly by the selective action of the dextran sulfate employed in this techniuqe.

This selectivity was also demonstrated by a comparative study carried out in our laboratory detailed elsewhere in this report. The viruses employed were laboratory strains of poliovirus 1, echovirus 7 and "natural" viruses. The natural viruses were, in fact, small amounts of domestic sewage that were introduced into the sample. The pronounced difference in the recovery efficiency of laboratory strains and "natural" virus was striking. Recovery of laboratory strains was satisfactory to very good while "natural" viruses provided a relatively low recovery. It should be remembered that these "natural" viruses are present in the water sources and it is their isolation that we that we want to achieve. It is therefore important that virus concentration methods be examined as to their ability to concentrate not only laboratory strains but also, and specifically, the "natural" virus, as was done by us.

THE ELUENT AND RECOVERY EFFICIENCY

Another outstanding fact is the discrepancy between input and recovered virus. The quantity of the recovered virus at times is larger than the inoculum. The phenomenon cannot be ascribed to multiplication since viruses multiply in cells only, contrary to bacteria. In trying to elucidate this finding, the question of the different eluates in the methods was raised. And indeed, suspension of the control virus in the various eluates sufficed to increase the titer 2 times, and sometimes even more. Hamblet et al[35] also describe similar findings.

The relationship between inoculum and recovered virus is probably connected with the existence of virus clumps, whose dissociation is caused by the eluent. Such virus clumps were described by Galasso and Sharp.[36] Virus clumps are important in the monitoring of water when exact quantitative results are desired. Faulty quantitation caused by such clumps may have serious consequences. It should be stressed therefore that such clumps have to be taken into account in the concentration of viruses from water.

WATER QUALITY AND THE EFFICIENCY OF THE METHODS

In nature viruses are found in waters of different qualities: raw sewage, waste water effluents at various stages of treatment, rivers, lakes, and seawater. These waters may contain suspended particulates, organic matter or salts. Their pH may vary from alkaline to acid. All these factors affect the virus concentration methods, specifically the large adsorption and elution group. In these latter methods, adsorption takes place as a result of electrostatic attraction between the adsorbant and the viruses. Particulates, organic matter, salts and pH differences affect the adsorption efficiency. Berg et al [37] described the strong inhibitory effect of a very small amount of organic matter on the recovery efficiency of the cellulose nitrate membrane adsorption method. Wallis et al,[32] on the other hand, showed that the addition of a small quantity of aluminum chloride increases the efficiency of this particular method, and Pao and co-workers [38] were able to fully recover inoculated entero-virus from raw sewage that had been sterilized by simply lowering its pH to 3.0. On the basis of these results, Wallis et al [39] incorporated a special procedure into their portable virus concentrator, which automatically lowers the pH of the sample. Efficient use of the equipment is hampered, however, by the need for adjustment with each change of water quality.

Another aspect of water quality is the presence of suspended solids. These solids play an important role in methods involving filtration since they clog the filters. In our laboratory we did not succeed in filtering more than 10 liters of prefiltered water through an alginate filter with a 142 mm diameter. Results were even worse with ordinary tapwater: not more than 5 liters could be passed through one filter.

The quantity of water necessary for good virological evaluation is a moot point. Berg[40] suggested a minimal volume of 100 gallons. In the light of current developments of concentration methods which enable the processing of such large volumes of water, this is not an exaggerated demand.

In trying to find a solution for the clogging of the filters, a prefiltration step was added to some of the methods. In this context Wallis et al[39] suggested to serially pass the water through clarifying filters with porosities of 1-5 μm to remove particulate matter and then through a 1 μmeter cotton textile filter to electrostatically remove submicron ferric and other heavy metal complexes. In experiments carried out under controlled laboratory conditions, the authors succeeded in recovering 80% of the virus that had been added to large quantities of water. It is reasonable to assume that under field conditions, however, recovery efficiency will fall far below the above number. This assumption rests on Berg's[41] finding that in natural environment more than 50% of the viruses are sometimes adsorbed onto solids, or manifest themselves in the form of large clumps. Prefiltration may extract these viruses, and a false picture of the quantity of virus in the water is thus obtained.

SAMPLE VOLUME AND RECOVERY EFFICIENCY

Some workers maintain that recovery of the entire virus population from the water is superfluous and, anyhow, almost impossible to achieve.

Sampling of large volumes, even when recovery will be low, should compensate for this flaw.

The gauze pad method, advocated by many in this connection, is easy to use and facilitates large water volumes to be sampled. Some researchers[27,42] pointed to the feasibility of using the gauze pad under extreme conditions. In its original conception the method was meant to be qualitative only. Coin,[43] however, built a flow-through gauze sampler which allows a quantitative virus estimation as well. Liu et al[44] tested virus recovery with a similar apparatus and came to the conclusion that in spite of its low recovery, a further development of this device appears warranted since (a) the method is simple, (b) it is capable of sampling large volumes of water, (c) the cost of collecting samples is low, and (d) it enables a rough quantitative assessment of viral pollutants in water.

This last proposition was examined in our laboratory with the aid of a flow-through gauze sampler similar to the one described by Liu et al.[44] We tested the effect of sample volume on the recovery efficiency of the method. In Section V, we show that there is an inverse relationship between sample volume and virus recovery: the larger the former, the lower the latter. It follows that the method is unsuitable for quantitative virus estimation.

THE TIME FACTOR IN VIRUS ISOLATION

The most prominent reason for monitoring viruses in water is to protect the population from health hazards. The results of the tests should therefore be available before the water reaches the consumers. Since impoundment of vast quantities of water for long periods of time is undesirable, rapid results of water testing is therefore imperative.

Standard bacteriological tests take 24 hours, and even this relatively short time is too long. What should be said, then, about virological tests which take days, and sometimes even weeks? The reason for this time lag is not inherent in the concentration methods. Although some methods are slow to perform, as for example, the phase-separation method which needs 24-48 hours,[45] most concentration methods require 1-6 hours only. The time taken up in virus isolation, on the other hand, is much longer. The accepted techniques involve inoculation of tissue cultures followed by incubation. Virus is then demonstrated by the appearance of cytopathic effect or plaques in the tissue cultures. The entire process lasts from 4 to 7 days, or longer, depending on the method. In our laboratory, the plaque assay is used and 5 days after sampling quantitative evaluation of virus is possible. The time needed for plaque assay can be shortened, but then the danger exists of incomplete plaque development, which is expressed by a too small number of plaques or by false negatives.

In an effort to find a solution for the difficulties related to the time problems, a project for the rapid isolation and identification of viruses from water by a fluorescent antibody (FA) technique was initiated in our laboratory, and is reported in Section VIII. Fluorescent antibodies stain cells that contain viral antigens and thus enable identification of eventual viruses in the cells considerably before the development of a visible cytopathic effect. We have shown that with this method it is possible to achieve qualitative results in 9 hours and a quantitative virus assay in 18-24 hours.

CONCLUSIONS

In this review only part of the problems related to virus monitoring in water were dealt with. Items such as cost and simplicity of the methods were barely mentioned, although these are important factors. The aspects discussed here are characterized by their direct effect on the ability of the monitoring methods to quantitatively and rapidly isolate the great variety of viruses found in large volumes of water of different qualities.

Some of the problems are relatively easy to solve. For example, the inverse effect of the sample volume on the recovery efficiency is characteristic for the gauze method only. It was mentioned here to serve as a warning to be taken into account when developing new methods. On the other hand, clumps and viruses adsorbed onto solids are intricate matters. Comprehensive research is required to determine how far these factors are implicated in the quantitative estimation of viruses in water.

Although many concentration methods are available, none of them can be said to be superior in the full sense of the word. Many workers have directed their research activities toward adsorption and elution methods, since these are easy to perform. But these methods are sensitive to changes in water quality. An answer to this problem may be the latest ultrafiltration methods such as reverse osmosis. In our laboratory we are testing the ability of cellulose acetate hollow fiber units to concentrate viruses from water (See Section VII). These units provide large filtration areas in relatively small space. One of the units tested is approximately 5 cm in diameter and 50 cm in height. In spite of its small size, it dehydrates at the rate of about 500 ml per minute. This means that 100 liters are reduced into a reasonably small volume in less than 4 hours. Since these units are not affected by salinity, pH differences or organic matter, the method is promising and continued research into this direction is recommended.

The need for quick results of virological tests cannot be underestimated. Somehow, this subject has not been given the attention it deserves and as a result it lags behind. This is apparently one of the reasons why virological examinations are not accepted as required tests for potable water. Interest should be focused on the time factor and priority be given to research in this area. Our findings with the fluorescent antibody technique point toward a possible solution.

The problem of isolation of the numerous virus types and their recovery still remains insurmountable. It is unlikely that a single system, sensitive enough to isolate the great variety of viruses in water will be discovered in the near future. In this context it should be noted that coliform bacteria are used as indicators for fecal pollution of water. These bacteria are very well suited for this purpose since they are always present in feces and, consequently, in raw domestic sewage. Of the great number of enteroviruses, only the 3 polio types are always present in domestic sewage of urban areas in developed countries because of the widespread routine administration of live polio virus vaccine to infants. It would be advisable, therefore, to weigh the possibility of having these viruses serve as indicators of viral pollution of water. It may be added that such choice will certainly solve many of the problems related to the monitoring of viruses in water.

REFERENCES

1. Mosley, J.W. Transmission of Viral Diseases by Drinking Water. In: Transmission of Viruses by the Water Route, Berg, G. (ed.). London, Interscience, 1962.

2. Bancroft, P.M., W.E. Engelhard and C.A. Evans. Poliomyelitis in Huskerville (Lincoln), Nebraska. J. Am. Ass. 164:836, 1957.

3. Dennis, J.M. Infectious Hepatitis Epidemic in New Delhi India. J. Am. Wat. Wks. Ass. 51:1288-1296, 1959.

4. Clarke, N.A. and P.W. Kabler. Human Enteric Viruses in Sewage. Hlth. Lab. Sci. 1:44, 1964.

5. Shuval, H.I. Detection and Contrul of Enteroviruses in the Water Environment. In: Development in Water Quality Research, Shuval, H.I. (ed.). Ann Arbor, Ann Arbor Humphreys Publ. 1970.

6. Plotkin, S.A. and M. Katz. Minimal Infective Doses of Virus for Man by the Oral Route. In: Transmission of Viruses by the Water Route, Berg, G. (ed.), London, Interscience, 1967.

7. Kjellander, J. and E. Lund. Sensitivity of E.coli and Polio virus to Different Forms of Combined Chlorine. J. Am. Wat. Wks. Ass. 57:893, 1965.

8. Shuval, H., S. Cymbalista, A. Wach, Y. Zohar and N. Goldblum. The Inactivation of Enteroviruses in Sewage by Chlorination. Proc. 3rd Int. Conf. Water Pollution Res., January 1967.

9. Kissling, R.E. Laboratory Status of the Infectious Hepatitis Agent. In: Transmission of Viruses by the Water Route, Berg, G. (ed.). London, Interscience, 1967.

10. Enders, J.F., T.H. Weller, and F.C. Robbin. Cultivation of the Lansing Strain of Poliomyelitis Virus in Cultures of Various Human Embryonic Tissues. Science.109:85, 1949.

11. Rawal, B.D. and S.H. Godbole. A Simple Tissue Culture Method for Detection of Viruses in Drinking Water; Sample Incorporation Method. Environmental Health. 6:234, 1964

12. Berg, G., D. Berman, S.L. Chang, and N.A. Clarke. A Sensitive Quantitative Method for Detecting Small Quantities of Virus in Large Volumes of Water. Am. J. Epiol. 83:196, 1966.

13. Gartner, H. Retention and Recovery of Polioviruses on a Soluble Ultrafilter. In: Transmission of Viruses by the Water Route, Berg, G. (ed.). London, Interscience, 1967.

14. Shohmair, K. Virus Concentration by Ultrafiltration. In: Methods in Virology, Maramorosk, K.and H. Kopewski, (eds.). New York, Academic Press, 1967. Vol. II:245-274.

15. Shuval, H.I., S. Cymbalista, B. Fattal and N. Goldblum. Concentration of Enteric Viruses in Water by Hydro-Extraction and Two-Phase Seperation. In: Transmission of Viruses by the Water Route, Berg. G. (ed.). London, Interscience, 1967.

16. Cliver, D.O. Detection of Enteric Viruses by Concentration with Polyethylene Glycol. In: Transmission of Viruses by the Water Route, Berg. G. (ed.). London, Interscience, 1967.

17. Sweet, B.H., J.S. McHale, K.S. Hardy, F. Morton, J.K. Smith, and E. Klein. Concentration of Virus from Water by Osmotic Ultra-Filtration. Water Res. 5:823-829, 1971.

18. Rubenstein, S.H., H.G. Orbach, N. Shuber, E. King and J. Zachler. Freeze Concentration of Viral Agents from Large Volumes of Water. J.A.W.W.A. 63:301-302, 1971.

19. Albertson, P.A. Partical Fractionation in Liquid Two-Phase Systems. Biochim. et Biophys. Acta. 27:378, 1958.

20. Albertson, P.A. Partition of Proteins in Liquid Polymer Two-Phase Systems. Nature. 182:709, 1958.

21. Frick , G. and P.A. Albertson. Bacteriophage Enrichment in a Liquid Two-Phase System with a Subsequent Treatment with "Freon" 113. Nature. 183:1070, 1959.

22. Anderson, N.G., G.B. Cline, W.W. Harris and J.G. Green. Isolation of Viral Particles From Large Fluid Volumes. In: Transmission of Viruses by the Water Route, Berg. G. (ed.). London, Interscience, 1967.

23. Brinton, C.C. and M.A. Lauffer. In: Electrophoresis, Bier, M. (ed.). New York, Academic Press, 1959.

24. Bier, M., G.C. Bruckner, F.C. Cooper and H.E. Roy. Concentration of Bacteriophage by Electrophoresis. In: Transmission of Viruses by the Water Route, Berg, G. (ed.). London, Interscience, 1967.

25. Ellender, R.D., F. Morton, J. Whelan and B.H. Sweet. Concentration of Virus from Water by Electro-Osmosis and Forced-Flow Electrophoresis. Prep. Biochem. 2:215-220, 1972.

26. Sweet, B.H. and R.D. Ellender. Electro-Osmosis: A New Technique for Concentrating Viruses from Water. Water Res. 6:775-779, 1972.

27. Melnick, J.L., J. Emmons, E.M. Opton and J.H. Coffey. Coxsackie Viruses from Sewage. Methodology Including an Evaluation of Grab Sample and Gauze Pad Collection Procedures. Am. J. Hyg. 59:185-195, 1954.

28. Wallis, C. and J.L. Melnick. Concentration of Viruses on Aluminum and Calcium Salts. Am. J. Epidemiol. 85:459. 1966.

29. England, B. Concentration of Reovirus and Adenovirus from Sewage and Effluents by Protamine Sulfate Treatment. Appl. Microbiol. 24:510-512, 1972.

30. Wallis, C., S. Grinstein, J.L. Melnick and J.E. Field. Concentration of Viruses from Sewage and Excreta on Insoluble Polyelectrolytes. Appl. Microbiol. 18:1007, 1969.

31. Cliver, D.O. Factors in the Membrane Filtration of Electroviruses. Appl. Microbiol. 13:412, 1965.

32. Wallis, C., M. Henderson and J.L. Melnick. Enterovirus Concentration on Cellulose Membranes. Appl. Microbiol. 23:476-480, 1972.

33. Nupen, E.M. Virus Studies on the Windhoek Wastewater Reclamation Plant. Water. Res. 4:661-672, 1970.

34. Grindrod, J. and D.O. Cliver. Limitations of the Polymer Two-Phase System for Detection of Viruses. Arch. Ges. Virusforsch. 28:337-347, 1969.

35. Hamblet, F.E., W.F. Hill, Jr., and E.W. Akin. Effect of Plaque Assay Diluent upon Enumeration of Poliovirus Type I. Appl. Microbiol. 15:208, 1967.

36. Galasso, G.L. and D.G. Sharp. Virus Aggregation and the Plaque-Forming Unit. J. Immunol. 88:339, 1962.

37. Berg, G., D. R. Dahling and D. Berman. Recovery of Small Quantities of Viruses from Clean Waters on Cellulose Nitrate Membrane Filters. Appl. Microbiol. 22:608-614, 1971.

38. Rao, V.C., U. Chandorkar, N.U. Rao, P. Kumaran and S.B. Lakhe. A Simple Method for Concentrating and Detecting Viruses in Wastewater. Water Res. 6:1565-1576, 1972.

39. Wallis, C., A. Hanna and J.L. Melnick. A Portable Virus Concentrator for Testing Water in the Field. Water Res. 6:1249-1256, 1972.

40. Berg, G. An Integrated Approach to the Problem of Viruses in Water. In: Proc. of the National Specialty Conference on Disinfection. New York, A.S.C.E., 1971. p339-364.

41. Berg, G. Reassessment of the Virus Problem in Sewage and in Renovated Waters. In: Progress in Water Technology, Jenkins, S.H. (ed.). New York, Pergamon Press, 1973. Vol. 3, p. 87-94.

42. Lund E., and C.E. Heckström. A Study on Sampling and Isolation Method for the Detection of Viruses in Sewage. Water Res. 3:823-832, 1969.

43. Coin, L. In: Transmission of Viruses by the Water Route, Berg, G. (ed.). New fork, Wiley Interscience, 1967.

44. Liu, O.C., D.A. Brashear, H.R. Seraichekas, J.A. Barwick and T.G. Metcalf. Viruses in Water. Appl. Microbiol. 21:405-410, 1971.

45. Shuval, H., B. Fattal, S. Cymbalista and N. Goldblum. The Phase-Separation Method for the Concentration and Detection of Viruses in Water. Water Res. 3:225-340, 1969.

SECTION V

COMPARISON OF EIGHT CONCENTRATION METHODS FOR ISOLATION OF VIRUSES IN WATER

INTRODUCTION

Various methods for the detection of small quantities of viruses in water have recently been developed. Hill et al[1] and Shuval and Katzenelson[2] have reviewed and compared different individual studies of methods for concentration and detection of viruses in water. However, for proper evaluation of different methods, it is essential to use the same water sample in controlled tests of recovery efficiency. Different research workers have reported studies in which two or more concentration procedures were compared on split samples. The results of these studies are summarized by England.[3]

This study presents a controlled comparison of eight different methods for the concentration and detection of seeded enteroviruses in a known water sample and is aimed to determine which of these method(s) would be most appropriate for routine monitoring of polluted water or renovated wastewater.

MATERIALS AND METHODS

Viruses

Attenuated poliovirus I was obtained and treated as described by Shuval et al.[4] Echovirus 7 (Wallace, AGKP 8A), was obtained from G. Berg, Environmental Protection Agency, Cincinnati, Ohio. Both viruses were stored in 2 ml volumes at -70°C, thawed shortly prior to each experiment, and diluted in M-199 medium to the concentration required.

Cell Cultures and Virus Assays

Vero Cells -

A continuous line of African green monkey kidney cells (Flow Laboratory Ltd., Scotland) was used for studies with poliovirus. The cells were grown in M-199 medium, supplemented with 10% calf serum.

BGM cells -

A continuous line of monkey kidney cells (kindly supplied by Dr. G. Berg) was grown in M-199 medium supplemented with 10% fetal bovine serum. These cells have been characterized by Barron et al[5] and Dahling et al.[6] The cells were used in experiments with echovirus 7 and with viruses from diluted raw sewage. Virus was titrated by the plaque method, using disposable plastic petri dishes, and was expressed as plaque forming units (pfu). Virus assays were carried out as described by Shuval et al;[4] the incubation period was 3 days.

Antibiotic Solution

One ml of antibiotic stock solution contained 200 mg streptomycin, 2.0×10^5 units of penicillin, 4 mg neomycin and 5 mg kanamycin. The solution was sterilized by filtration. All media or solutions used in this study contained 1 ml of antibiotic solution per liter unless otherwise stated.

Aluminum Hydroxide

The suspension was essentially prepared as described by Wallis and Melnick.[7] However, the stock suspension was twice as concentrated as the above described, according to B. England (personal communication).

Membrane and Filtration Equipment

These consisted of pressure vessels and filter holders of stainless steel. Filters used were either type MF (Millipore Corp), 47 mm diameter, 0.45μ pore size, or Sartorius (Cat. #SM 11306), 142 mm diameter, 0.45μ pore size, or aluminum alginate Sartorius ultra filters (Cat. # 12710), 142 mm diameter, pore size 0.05μ. For some experiments Gelman Cartridge filters were used: (Cat. No. #12104, 3μ pore size and Cat. No. 12106, 0.22μ pore size, both 16 cm length).

Ultrasonic Treatment

This was carried out with the aid of a Measiring and Scientific Equipment Ltd., London, MSC 100 watt ultrasonic disintegrator, probe #25925, using a 20 ml tube at maximum power and peak activity for 10 min.

Gauze Sampler

A special gauze sampler was constructed for this study as described by Fattal et al[8] (See also Section VII).

Concentration Methods and Procedures

This study was carried out in two stages: the first was to compare five different methods for the concentration and detection of seeded virus in 5 equal aliquots of the same tapwater sample. The procedures of these methods are described in test procedure A. The second stage was to compare 4 methods one of which had been employed in stage one. This stage is described in test procedure B. In both stages, we used the aluminum hydroxide precipitation method (Wallis and Melnick[7]) thus enabling us to compare methods from the two stages.

Test Procedure A-

A typical experiment was carried out as follows: 25 liters of tap water were dechlorinated with 15 ml of 10% sodium thiosulfate, the pH adjusted to 7.0, and then filtered through a membrane filter (Millipore Corp) with porosity of 0.45µ. The virus stock was diluted in M-199 medium and added to the water sample until the desired concentration was attained. The sample was divided into five equal aliquots, each of which was treated by one of the methods described below. The virus stock diluted in the M-199 medium and assayed for viruses served as control.

1. Aluminum Alginate Ultrafiltration - (according to Gartner[9]). Water samples of 5 liters were filtered through an aluminum alginate ultra filter at a positive pressure of 2 atmospheres. After filtration, the membrane was separated from the supporting filter and dissolved in 5 ml sterilized 3.8% citrate solution and 0.5 ml antibiotic solution. The final volume was 6 ml of concentrate.

2. Cellulose Nitrate Membrane Filtration - (according to Berg et al[10]). A 5 liter sample was passed through a cellulose nitrate membrane filter (Millipore Corp) at a negative pressure of about 700 mm Hg. Each filter was then homogenized in a Sorval Omni-Mixer (# 17106) at high speed for 5 min with 10 ml of a 3% Difco beef extract solution and sonicated. The suspension was centrifuged at 250 x g for 5 min and decanted. One ml antibiotic solution was added to the supernatant. The final volume was 12 ml of concentrate.

3. Aluminum Hydroxide Precipitation - (according to Wallis and Melnick[7]). Aluminum hydroxide suspension (12 ml) was added to a 5 liter sample. The mixture was stirred gently with a magnetic stirrer for 60 min at room temperature and then filtered by positive pressure through a Sartorius filter. The precipitate was trapped on the filter, recovered with a spatula and suspended in 5 ml saline containing 0.5 ml antibiotic solution. The final volume was 5.6 ml of concentrate.

4. Phase Separation - (according to Shuval et al[11]). To a 5 liter water sample, the following compounds were added: 10 gr sodium dextran sulfate 500 (Pharmacia, Sweden), 322 gr polyethylene glycol (Carbowax 6,000) and 105 gr sodium chloride. The mixture was stirred with a magnetic stirrer, transferred to a separatory funnel and kept overnight at 5°C. The lower- and interphase were removed; their combined volume averaged 35 ml. To each milliliter, 0.3 ml of 3 M KCl was added and the solution centrifuged at 15,000 x g for 30 min. One ml antibiotic solution was added to every 10 ml of supernatant. The final volume was 35 ml of concentrate.

5. Flow-through Gauze Sampler - A 5 liter water sample was filtered through the gauze sampler at a positive pressure of about 0.1 atmosphere. After filtration, the gauze pad was removed from the sampler with the aid of tweezers and transferred into a plastic bag. The pH was adjusted to 8.0 with a 1% NaOH solution. The water in the pad was measured and calf serum was added to a final concentration of 5%. The pad was then hand-pressed 5-7 times. The pad, placed in a specially designed polypropylene insert, as described by Fattal et al,[8] with a perforated bottom, was placed in a standard 250 ml centrifuge tube (see Section VII in this report) and centrifuged at 4,000 x g for 10 min. Nearly all of the water content in the pad could thus be recovered. The dry pad was replaced in the plastic bag and 20 ml saline, pH 8.0, containing 5% calf serum were added. The processes of absorption, expression and centrifugation were repeated to obtain a second eluate; the liquid from this elution was mixed with that of the first one. The average volume of the combined eluates was 82 ml. One ml antibiotic solution was added to every 10 ml of eluate. The final volume was 90 ml of concentrate.

The five different concentrates and the diluted control virus were kept at 4°C and assayed the following day, or kept at -70°C until assayed. Nearly the entire volume of each concentrate using the first three methods was assayed. However, only about one third of the volume of the concentrates of the fourth method and about one quarter of the fifth method were assayed.

Test Procedure B -

Twenty liters of tapwater were dechlorinated, pH adjusted, prefiltered and virus added, as described in test procedure A. The sample was divided into four equal aliquots, each of which was treated by one of the following methods:

1. Aluminum Hydroxide Precipitation - as described in test procedure A.

2. Cellulose Nitrate Membrane Filter - (according to Wallis et al[12]). A 5 liter sample was adjusted to pH 3.5 with HCl and then $AlCl_3$ was added to give a final concentration of 0.0005 M $AlCl_3$. The sample was filtered through a 47 mm cellulose nitrate membrane filter (0.45μ pore size). The membrane was then washed with 5 ml of saline at pH 7.0 and then eluted with 5 ml of 0.05M glycine buffer at pH 11.5. Five ml of glycine buffer, 0.05M pH 2 neutralized the previous buffer. One ml antibiotic solution was added. The final volume was 11 ml of concentrate.

3. Insoluble Polyelectrolytes (PE 60) - (according to Wallis and Melnick[13]). A 5 liter sample was adjusted to pH 5.0 with HCl and then filtered through polyelectrolyte layers of 47 mm diameter. The PE 60 pad layers were prepared by adding 2 ml of a 10% suspension of PE 60 (prepared as described by Wallis et al[7]) to 300 ml of H_2O stirred with the aid of a magnetic stirrer for 5 min and filtered by negative pressure through an AP 20 pad. The layer contained 100 mg of PE 60. After filtration, the virus which was adsorbed to the layer, was eluted with 10 ml of 0.05 M borate buffer pH 9.0 containing 10% bovine calf serum.

One ml of antiobiotic solution was added. The final volume was 11 ml of the concentrate.

6. Cellulose Nitrate Membrane Filter - (eluting method according to Rao and Labzoffsky[14]). A 5 liter sample was adjusted to pH 3 with HCl and then filtered through a 47 mm cellulose membrane 0.45μ pore size. Ten ml of a sterile 3% solution beef extract pH 8 was added to the membrane to elute the virus. One ml of antiobiotic solution was added to eluate. The final volume of the concentrate was 11 ml.

Concentration of Attenuated Poliovirus I By Gelman Membrane Filters

In addition to a comparison of different concentration methods, some experiments were carried out with the aid of Membrane Cartridge filters supplied by Gelman, using water samples of between 160-360 liters in 40 liter aliquots. Each 40 liter sample was dechlorinated and the pH adjusted to 3.5 with concentrated HCl. The virus stock was diluted in M-199 medium and added to the water sample until the desired concentration was attained. The sample was filtered through the Cartridge filter by a positive pressure of 0.5-1.5 PSI. The flow rate was 1.2 liter per min. The virus was eluted from the cartridge by adding 80 ml of 0.05 M glycine buffer at pH 11.5. The pH was then immediately adjusted to 7.0 with an HCl solution. Eighty ml of 0.05 M glycine buffer at pH 11.5 were again added to the filter for a second and third elution. The ph was adjusted to 7.0 with HCl. One ml of antiobiotic solution was added to each 9 ml of eluate. The final volume of the three eluates were 300 ml.

RESULTS

Recovery of Attenuated Poliovirus I

The results of the recovery efficiency of attenuated poliovirus I in 5 liter water samples are summarized in Tables 1, 2 and 3. Table 1 shows the recovery for four methods described in test procedure A of virus concentrations at low input levels (8-64 pfu/5 liters). Table 2 describes the recovery for five methods from test procedure A with an input of 390-790 pfu/5 liters. Table 3 shows the recovery for methods described in test procedure B, with virus inputs of 40-827 pfu/5 liters.

There are only slight differences among the recovery efficiencies of the first four methods, while that of the gauze pad is extremely low (about 1%). It should be pointed out that the results for the gauze pad method were not presented in Table 1 because of its very low concentration factor and recovery. By employing this method, viruses would not be detectable when found in such low concentrations as were used in experiments in Table 1.

Table 1 clearly shows the high recovery of the alginate filter and of the aluminum hydroxide method. A recovery of over 100% in each experiment is particularly noticeable, with an average of 247% for the alginate and 213% for the aluminum hydroxide method. The average recovery with the cellulose nitrate method,according to Berg et al [10], was 63%. The phase separation method shows an average recovery of more than 100% while that of 5 out of 7 experiments was more than 80%. With all four methods, 8 pfu/5 liters could be detected.

Table 1. THE RECOVERY EFFICIENCY OF FOUR METHODS FOR THE CONCENTRATION OF SEEDED ATTENUATED POLIOVIRUS 1 AT LOW INPUT LEVELS IN TAP WATER

Exp. no.	Calculated total poliovirus input pfu/5 ℓ	% Virus recovered			
		Alginate filter	Cellulose nitrate MF (Berg et al[10])	$Al(OH)_3$	Phase separation
1	8	160	18	111	169
2	10	243	100	263	43
3	15	226	63	105	41
4	16	258	71	236	242
5	27	464	72	284	123
6	35	266	70	366	85
7	64	101	45	127	81
Ave.	8-64	287	63	213	112
S.D.		±113	±26	±101	± 73

Table 2. THE RECOVERY EFFICIENCY OF FIVE METHODS FOR THE CONCENTRATION OF SEEDED ATTENUATED POLIOVIRUS I AT HIGH INPUT LEVELS IN TAP WATER

Exp. no.	Calculated total poliovirus input pfu/5 L	% Virus recovered				
		Alginate filter	Cellulose nitrate MF (Borg et al[10])	$Al(OH)_3$	Phase separation	Gauze pad
1	391	44	36	93	68	0.4
2	567	86	58	89	63	1.0
3	792	165	107	176	89	1.5
Ave. S.D.	390-790	98 ±61	67 ±36	119 ±49	73 ±22	0.97 ±0.55

Table 3. THE RECOVERY EFFICIENCY OF FOUR METHODS FOR THE CONCENTRATION OF SEEDED ATTENUATED POLIOVIRUS I AT VARIOUS INPUT LEVELS IN TAP WATER

Exp. No.	Calculated total poliovirus input pfu/5 L	% Virus recovered Al(OH)$_3$	MF[a] (Rao et al)	MF[b] (Wallis et al)	PE 60
1	40	65	93	53	83
2	54	30	57	48	65
3	101	70	101	119	69
4	101	99	89	57	55
5	153	53	167	29	144
6	180	30	62	107	74
7	183	-	36	56	40
8	320	23	29	51	40
9	322	23	65	44	53
10	827	60	67	83	76
Ave. S.D.	40-887	50 ±25	77 ±40	65 ±29	70 ±30

[a] MF = Cellulose nitrate-elution according to the Rao et al method[14]

[b] MF = Cellulose nitrate according to Wallis et al[12]

Table 2 indicates an average recovery of about 100% with the alginate filter and aluminum hydroxide as compared to the average recovery of about 70% with cellulose nitrate and phase separation. The gauze pad method shows a very low recovery of about 1%.

Table 3 shows that the recovery efficiency of MF according to Rao et al.[14] is higher than the others. The average recovery with $Al(OH)_3$ was 50%. The MF Rao method shows an average recovery of 77% while that of the MF Wallis et al method[12] was 65% and PE 60-70%.

The Effect of Sample Volume on Recovery Efficiency of the Gauze Pad Sampler

To obtain a better understanding of the mechanism by which the gauze sampler concentrates viruses, the effect of sample volume on attenuated poliovirus I was tested. As can be seen in Table 21, Section VII, virus recovery is dependent on sample volume. In quantities of 60 liters, recovery averaged 0.5%, and in volumes of 700 ml it was 7%. The larger the volume, the lower the efficiency of the pad.

Table 21 shows the sharp decrease in recovery efficiency relative to the sample volume in each experiment. On the other hand, the concentration factor of the gauze is obtained by dividing the pfu/ml of gauze fluid after filtration by the pfu/ml of the control fluid before filtration. This factor increases in direct proportion to the sample volume: the factor is 1.2 for 700 ml versus 6.6 for 51,600 ml. The experiments were performed simultaneously. Each gauze pad was eluted 4 times and centrifuged; the recovery efficiency is the total of these four elutions.

Recovery of Echovirus 7

Since poliovirus is only one of the various viruses found in sewage, an additional enterovirus present in sewage, echovirus 7, was also tested. Table 4 shows the results of virus recovery by the five methods described in test procedure A for concentration of seeded echovirus 7 at various input levels (5-2,000 pfu/5 liters).

The alginate filter and phase separation methods show higher recovery than the other methods (average 101% and 125%, respectively). Aluminum hydroxide shows slightly higher results than the cellulose nitrate filter method. It is worthwhile to mention that in Experiments 1 and 2, three methods were successful: alginate, $Al(OH)_3$ and phase separation, and we were able to detect 5 and 7 pfu/5 liters of water at good recovery efficiency. The gauze pad shows again an extremely low recovery, even with high virus concentration.

Recovery of Enteroviruses from Sewage Contaminated Water

A comparison of virus detection methods using laboratory virus strains is not complete unless supported by experiments with natural wild viruses usually found in wastewater. Eleven experiments, 4 performed in test procedure A, and 7 in test procedure B, were conducted in which raw sewage was added to tapwater samples. Two to 20 ml of domestic raw sewage were added to 20 liters of dechlorinated filtered tapwater,

Table 4 THE RECOVERY EFFICIENCY OF FIVE METHODS FOR THE CONCENTRATION OF SEEDED ECHOVIRUS 7 AT VARIOUS INPUT LEVELS IN TAP WATER

Exp. no.	Calculated total virus input pfu/5 L	% Virus recovered				
		Alginate filter	Cellulose nitrate MF (Berg et al[10])	$Al(OH)_3$	Phase separation	Gauze pad
1	5	202	< 8	24	177	N.D.[a]
2	7	59	< 7	32	112	N.D.
3	17	128	72	23	127	N.D.
4	21	97	48	129	239	N.D.
5	207	215	35	75	176	N.D.
6	323	69	8	73	74	1.5
7	478	45	16	52	80	< 0.6
8	783	45	11	60	68	< 0.3
9	2,000	50	7	20	79	0.1
Ave. S.D.	5-2,000	101 ± 67	22 ±25	54 ±35	126 ± 60	∿ 0.6

[a] not done

adjusted to pH 7.0. Four virus test procedures were then carried out. The results are presented in Tables 5 and 6.

Table 5 shows an average recovery in all four methods of less than 50%, with a slight advantage, in most experiments, for alginate and aluminum hydroxide methods. The lowest recovery is found with the Phase Separation method in all experiments. Table 6 shows a high recovery efficiency of MF according to Rao et al (average of 111%). On the other hand, the average recovery efficiency of PE 60 is 80% compared to 67% according to the MF Wallis method. An average of 40% was obtained with $Al(OH)_3$.

Table 5. RECOVERY EFFICIENCY OF FOUR METHODS FOR THE CONCENTRATION OF ENTEROVIRUSES FROM SEWAGE CONTAMINATED WATER

Exp. no.	Dilution of sample with sewage %	Sewage control before dilution pfu/ml	Calculated total input enteroviruses pfu/5 l	% Virus recovered			
				Alg.F.[a]	MF[b]	$Al(OH)_3$	phase separation
1	0.01	45	23	26	14	11	8
2	0.02	27	27	74	5	32	N.D.[c]
3	0.02	35	35	15	14	31	10
4	0.03	38	67	72	26	60	5
Ave. S.D.	-	-	23-67	47 ±31	15 ± 9	34 ±20	8 ±5

a Alg.F = Alginate filter

b MF = Cellulose nitrate, according to Berg et al[10]

c N.D. = not done

Table 6. RECOVERY EFFICIENCY OF FOUR METHODS FOR CONCENTRATION OF ENTEROVIRUSES FROM SEWAGE-CONTAMINATED WATER

No.	Dilution of sample with sewage %	Sewage control before dilution pfu/ml	Calculated total input entero-viruses pfu/5 L	% Virus recovered: $Al(OH)_3$	% Virus recovered: MF[a] (Rao et al method)	% Virus recovered: MF[b] (Wallis et al method)	% Virus recovered: PE 60
1	0.010	6	3	67	167	167	--
2	0.015	9	7	43	114	71	71
3	0.025	11	12	--	242	108	142
4	0.025	6	7	29	57	29	71
5	0.1	4	19	32	21	11	68
6	0.1	16	82	21	101	45	71
7	0.1	24	118	48	75	36	54
Ave.			3-118	40	111	67	80
S.D.				±17	±74	±54	±21

[a] MF = cellulose nitrate-elution according to Rao et al[14]

[b] MF = cellusiose nitrate according to Wallis et al[12]

Recovery of Seeded Attenuated Poliovirus 1 by Cartridge Filter

In order to concentrate and detect seeded virus from large volumes of water, we decided to test also Membrane Cartridge filters from Gelman Company. By this method we succeeded in filtering up to 360 liters of tapwater. Seven preliminary experiments were carried out with the use of 2 kinds of filters: six experiments with a 3μ pore size filter and one expuriment with a 0.22μ pore size filter. The results are summarized in Table 7. From this table it can be seen that the recovery efficiency of the 3μ filter is between 27-168% compared to 36% for the 0.22μ filter. The lowest number of seeded poliovirus was 3.8 x 10^2 pfu/360 liters, i.e. approximately 1 pfu/liter with a recovery efficiency of 58%.

Table 7. RECOVERY EFFICIENCY OF THE GELMAN CARTRIDGE FILTER FOR THE CONCENTRATION OF SEEDED ATTENUATED POLIOVIRUS I AT VARIOUS INPUT LEVELS IN TAPWATER.

Exp. no.	Filter no.	Sample volume Filtered (L)	Total poliovirus input pfu	% Virus recovered
1	[a] 12104	160	1.1 x 10^7	27
2	12104	200	9.2 x 10^6	45
3	12104	200	1.3 x 10^6	163
4	12104	200	1.1 x 10^6	75
5	12104	360	9.5 x 10^2	47
6.	12104	360	3.8 x 10^2	58
7	[b] 12106	200	1.0 x 10^6	36

[a] 12104 = pore size 3μ

[b] 12106 = pore size 0.2μ

DISCUSSION

The number of enteroviruses detectable in sewage is considerable; their concentration changes from month to month and from one community to another. With direct inoculation tests carried out in our laboratory on Jerusalem domestic sewage, up to 95,000 pfu viruses/liter were found. This means that the average total number of viruses in the sewage flow from Jerusalem, with a population of 250,000, might be approximately 3×10^{12} pfu/day. In spite of the biological, chemical and physical treatment methods and the various disinfection processes, viruses may at times be present in water supplies, albeit in small numbers. Contaminated rivers will most likely contain viruses in various concentrations during most of the year. The quantity of water actually ingested by the public in cities with populations of one million and more is in the range of thousands of cubic meters per day. A small number of viruses that pass through the treatment barriers may therefore constitute a potential health hazard, particularly if we bear in mind that even one pfu of virus is capable of producing infection in susceptible hosts.[16]

From the epidemiological point of view, it is important that sensitive methods for the detection of small quantities of virus in large volumes of water be developed. This would allow the use of a viral indicator as a test for pollution in routine monitoring of water. Such a system is particularly important in view of the fact that under certain conditions the conventional bacterial indicators of pollution may be absent in treated water which nevertheless can still carry the more resistant enteroviruses. These virus detection methods should be inexpensive and simple enough to allow their use in most routine water monitoring laboratories, and should be able to detect one virus in a relatively large volume of water, preferably more than 100 liters.

Many methods have been developed during the last few years. Several research workers[1,2,3] have reviewed and compared different methods for detecting viruses in water. They compared various parameters, such as the concentration factor, recovery efficiency and time required for each method. However, few controlled comparison studies of methods have as yet been described. The present study tries to fill this gap. Eight methods, used today, for the concentration and detection of small quantities of viruses in water were compared. Two virus strains (attenuated poliovirus I and echovirus 7) and enteroviruses from sewage were employed.

Table 8 summarizes Tables 1, 2, 4 and 5. It can be seen that the first four methods provide relatively good recovery of seeded poliovirus and echovirus in water samples as compared to the poorer recovery obtained from samples of diluted sewage.

This finding can possibly be explained by the selectivity of the various concentration methods which may not concentrate all virus types with equal efficiency. The virus concentration of the sewage samples used in calculating recovery efficiency was made by the direct inoculation method, which leads to the least possible loss of viruses. Grindrod et al[16] have already shown that the phase separation method concentrates viruses selectively. As for the MF method according to Berg et al[10] and the aluminum hydroxide method, it appears possible that the organic matter in the sewage may compete for adsorption sites, thus reducing the efficiency of these methods when used with contaminated water.

Table 8. THE AVERAGE RECOVERY OF SEEDED ENTEROVIRUS AND THE CONCENTRATION FACTOR OF FIVE METHODS WITH TAP WATER

Virus	pfu/5 L	No. of exp.	% Average virus recovered				
			Alg.F.[a]	MF[b]	Al(OH)$_3$	Phase separation	Gauze pad
Polio-Virus I	8-790	10	202	64	185	100	1.0[c]
Echo-virus 7	5-2000	9	101	22	54	125	0.8[d]
Entero-virus from sewage	23-57	4	47	15	34	5[a]	-
Average final volume			6	12	6	35	90
Average concentration factor			850	425	850	140	55

[a] Alg.F. = alginate filter

[b] MF = cellulose nitrate according to the Berg et al method[10]

[c] = 3 experiments

[d] = 4 experiments

The average recovery of the alginate filter, the aluminum hydroxide method and the phase separation method was high with poliovirus (100% and over), as compared to the MF cellulose nitrate method of Berg et al (64%), and the gauze pad (only 1%). The average recovery of the alginate filter and phase separation methods were high with echovirus 7 (more than 100%), while the MF cellulose nitrate method (Berg et al) and aluminum hydroxide methods gave relatively low recoveries (22% and 54%, respectively). The gauze pad was again extremely low (~0.5%). The average recovery of enteroviruses from sewage was low with 4 methods and very low with the phase separation (5%) and MF cellulose nitrate (15%) methods (Berg et al).

Comparing our results with those of other workers, we find that Gartner[9] succeeded in recovering 25% of 10^6 pfu/10 liter and 100% of 100 pfu/10 liter with an alginate filter, which is similar to our findings for low concentrations of poliovirus.

Although there were differences in the technique used for MF cellulose nitrate (Berg et al), our results with poliovirus and echovirus 7 correlate well with those obtained by Berg et al.[10] Comparing the results of the aluminum hydroxide method with those of Wallis and Melnick,[6] the latter recovered about 80% of 249 pfu / liter, using poliovirus, while we found an average of 50% (Table 9) to 185% (Table 8). The recovery with the phase separation method in this study correlates well with the results obtained by Shuval et al.[4]

The recovery of the gauze pad was extremely low compared to the other four methods, which is in keeping with the results reported by Huff et al[17] and Liu et al.[18] Furthermore, recovery efficiency becomes lower as sample volume increases. It follows that the method is unsuitable for quantitative virus estimation.

Table 9 summarizes Tables 3 and 6. It can be seen that the MF method according to Rao et al[14] and PE 60 method provide good recovery of seeded poliovirus as well as diluted sewage: 70% and over on the average. The MF method according to Wallis et al[12] provides an average recovery of 65-67%, in both seeded poliovirus and diluted sewage, while with PE 60 the average recovery is 70-80%. A comparison of the data from the 8 methods presented in Tables 8 and 9, clearly shows the advantage of the three methods: MF - Rao, MF - Wallis and PE 60--for both seeded and diluted sewage. The elution of the virus from MF according to Rao method seems to be superior to the others. Our results correlated with those obtained by Rao et al,[14] MF Wallis et al[12] and with Wallis et al.[13]

In all experiments, the concentration factor of the eight methods ranged from 55 to 850. However, the MF, PE 60 and one variation of the aluminum hydroxide method can achieve concentration factors higher than 1,000.

For technical reasons, the alginate and phase separation methods are suitable for concentrations of not more than 10 liters per sample.

The first method required 6 hours (at 3 atmospheres pressure) and the second 18-24 hours. On the other hand, the MF cellulose nitrate, the aluminum hydroxide and gauze pad methods can be used for filtering large volumes of water, 100 liters and more.

Table 9. THE AVERAGE RECOVERY OF SEEDED ENTEROVIRUS AND THE CONCENTRATION FACTOR OF FOUR METHODS WITH TAP WATER.

			% Average virus recovered			
Virus	pfu/5 L	No. of exp.	$Al(OH)_3$	MF[a] Rao et al	MF[b] Wallis et al	PE 60
Polio virus 1	40-827	10	50[c]	77	65	70
Enterovirus from sewage	3-118	7	40[d]	111	67	80
Average of final volume			6	11	11	11
Average of concentration factor			850	450	450	450

[a] MF = cellulose nitrate-elution according to Rao et al[14]

[b] MF = cellulose nitrate according to Wallis et al[12]

[c] = 9 experiments

[d] = 6 experiments

All the methods described here are simple and not expensive to employ. For the alginate, MF and $Al(OH)_3$ methods, pure water, free of suspended particles, is required, while with the phase separation, the gauze pad method and PE 60 contaminated water can be used as well.

Recovery of more than 100% was obtained in many experiments. This phenomenon may be explained by the presence of virus clumps in the sample, which may have been disaggregated during the concentration procedure[19,20] and is very similar to the effect of diluent upon enumeration of poliovirus as described by Hamblet et al.[21] They found that nutrient broth when compared with Hank's balanced salt solution and an assaying diluent gave four to six times more plaques under similar conditions. They postulated that nutrient broth promotes disaggregation of virus clumps rather than enhances host cell susceptibility.

The discrepancies found between the recovery efficiency using the $Al(OH)_3$ in the first and second stage tests may be explained by the fact that the experiments in the second stage were carried out two years after the first stage experiments. During this period the stock polio virus which had been kept at -20°C probably underwent aggregation thus in turn bringing about poorer recovery efficiency at the second stage. On the other hand, there were no significant differences in recovery efficiency between the 2 stages for the $Al(OH)_3$ concentration of the diluted sewage.

The experiments carried out with the Gelman membrane cartridge filter indicate that the filter affords a good recovery efficiency, and is suitable for filtering large volumes (up to 360 liters) of water.

Hill et al[22] who also used cartridge filters, though larger, succeeded in filtering 400 liters of water and recoverd 1-2 pfu poliovirus/20 liters water. The above group used a combined technique, i.e., membrane cartridge filter adsorption followed by an aqueous polymer two steps phase separation method according to Shuval et al.[11] In conclusion, the cellulose nitrate membrane filter and the aluminum hydroxide methods are preferable for volumes of about 100 liters of water, while cartridge filters are preferable for 400 liters. Clean and organic-matter free water is a prerequisite when using these methods. Large volumes of unclean water may be used with the PE 60 method which is suitable for filtration of large volume of water containing organic matter. This certainly applies to drinking and renovated water. However, for samples of contaminated water, the alginate and aluminum hydroxide methods can be used effectively provided smaller water samples are used to avoid rapid clogging of the filters.

Although many improvements in virus detection methods can be anticipated, we feel that these results imply that even at the present stage of development, routine monitoring of drinking water is feasible and can provide a considerable degree of public health protection.

REFERENCES

1. Hill, W.F., W.E. Akin, and W.H. Benton. Detection of Viruses in Water: A Review of Methods and Application. Water Res. 5:967-995, 1971.

2. Shuval, H.I., and E. Katzenelson. The Detection of Enteric Viruses in the Water Environment. In: Water Pollution Microbiology., Mitchell, R. (ed.), New York, Wiley and Sons, 1972. p.347-361.

3. England B. Recovery of Viruses from Waste and Other Waters by Chemical Methods. In: Symposium on Detection of Viruses in Waste and Other Waters, Berg., G. (ed.). Developments in Industrial Microbiology, 1975, Vol. 5, p. 174-183.

4. Shuval, H.I., A. Thompson, B. Fattal, S. Cymbalista, and Y. Wiener. Natural Virus Inactivation Processes in Seawater. J. San. Eng. Div. 97:587-600, 1971.

5 Barron, A.L., C. Olshevsky, and M.M. Cohen. Characteristics of the BGM Line of Cells from African Green Monkey Kidney. Archiv. fur die gesamte Virusforschung. 32:389-392, 1970.

6. Dahling, D.R., G. Berg, and D. Berman. BGM, a Continuous Cell Line More Sensitive than Primary Rhesus and African Green Kidney Cells for the Recovery of Viruses from Water. Health Laboratory Sciences. 11:275-282, 1974.

7. Wallis, G., and J.L. Melnick. Concentration of Viruses on Aluminum Phosphate and Aluminum Hydroxide Precipitates. In: Transmission of Viruses by the Water Route, Berg, G. (ed.). New York, Wiley and Sons, 1967. p. 129-138.

8. Fattal, B., E. Katzenelson, and H.I. Shuval. Comparison of Methods for Isolation of Viruses in Water. In: Virus Survival in Water and Wastewater Systems, Malina, J.F., Jr. and B.P. Sagik, (eds.). University of Texas at Austin, Center for Research in Water Resources, 1974. p. 19-30.

9. Gartner, H. Retention and Recovery of Polioviruses on a Soluble Ultrafilter. In: Transmission of Viruses by the Water Route. Berg, G. (ed.). New York, Wiley and Sons, 1967. p. 121-127.

10. Berg, G., D.R. Dahling, and D. Berman. Recovery of Small Quantities of Viruses from Clean Waters on Cellulose Nitrate Membrane Filters. Appl. Microbiol. 22:608-614, 1967.

11. Shuval, H.I., B. Fattal, S. Cymbalista and N. Goldblum. The Phase Separation Method for the Concentration and Detection of Viruses in Water. Water Res. 3:225-240, 1969.

12. Wallis, G., M. Henderson, and J.L. Melnick. Enterovirus Concentration on Cellulose Membranes. Appl. Microbiol. 23:476-480, 1972.

13. Wallis, G., and J.L. Melnick. Detection of Viruses in Large Volumes of Natural Waters by Concentration on Insoluble Polyelectrolytes. Water Res. 4:787-796, 1970.

14. Rao, N.V., N.A. Labzoffsky. A Simple Method for the Detection of Low Concentrations of Viruses in Large Volumes of Water by the Membrane Filter Technique. Canadian Jour. of Microbiol. 15:399-403, 1969.

15. Plotkin, S.A., and M. Katz. Minimal Infective Doses of Viruses for Man by Oral Route. In: Transmission of Viruses by Water Route, Berg, G. (ed.). New York, Wiley and Sons, 1967. p. 151-166.

16. Grindrod, J. and D.O. Cliver. Limitation of Polymer Two Phase System for Detection of Viruses. Arch Ges. Viruuforsch. 28:227-247, 1969.

17. Hoff, J.C., R.D. Lee, and R.C. Becker. Evaluation of Methods for Concentration of Microorganisms in Water. Water Hyg. Div. p. 37, 1971.

18 Liu, O.C., D.A. Brahsear, H.R. Seraichekas, J.A. Barnick, and T.G. Metcalf. Virus in Water: A Preliminary Study on Flow-through Gauze Sampler for Recovering Virus from Water. Appl. Microbiol. 21:405-410, 1971.

19. Galasso, G.L. and D.G. Sharp. Virus Aggregation and the Plaque-Forming Unit. J. Immunol. 88:339-347, 1962.

20. Gerba, C.P. and G.E. Schaiberger. Aggregation as a Factor in Loss of Viral Titer in Seawater. Water Res. 9:567-571, 1975.

21. Hamblet, F.E., W.F. Hill Jr., and E.W. Akin. Effect of Plaque Assay Diluent Upon Enumeration of Poliovirus Type I. Appl. Microbiol. 15:208, 1967.

22. Hill, W.F., E.W. Akin, W.H. Benton, and T.G. Metcalf. Virus in Water: Evaluation of Membrane Cartridge Filters for Recovering Low Multiplicities of Poliovirus from Water. Appl. Microbiol. 23:880-888, 1972.

SECTION VI

VIRUS CONCENTRATION USING HOLLOW FIBER MEMBRANES

INTRODUCTION

The purpose of this study was to determine the ability of cellulose acetate hollow fibers, commonly used as an ultrafiltration membrane for biological applications[1] to concentrate viruses. The well known advantages associated with the fiber membrane permeators include compactness, i.e. very high active membrane surface area per unit volume of permeator, light weight, low water hold-up and low cost.[2] Virus concentration has been successful using flat ultra- and hyper-filtration membranes.[3,4] The advantages of the method as compared to others are no need to adjust pH and lack of interference by organic matter or salinity. It is the compactness, light weight and low water hold-up of the fiber unit that renders it attractive for the first (concentration) step in the detection of virus from large volumes of water. Once the viruses are concentrated into small volumes, the second step (virus enumeration) can be used to quantitate virus levels.

In this section, we present the results of an experimental study to concentrate viruses using a hollow fiber membrane permeator. The effect on permeator performance of the following independent variables, direction of flow through the fiber, feed flow rate, initial virus concentration in the feed, cell type, and backwashing was studied.

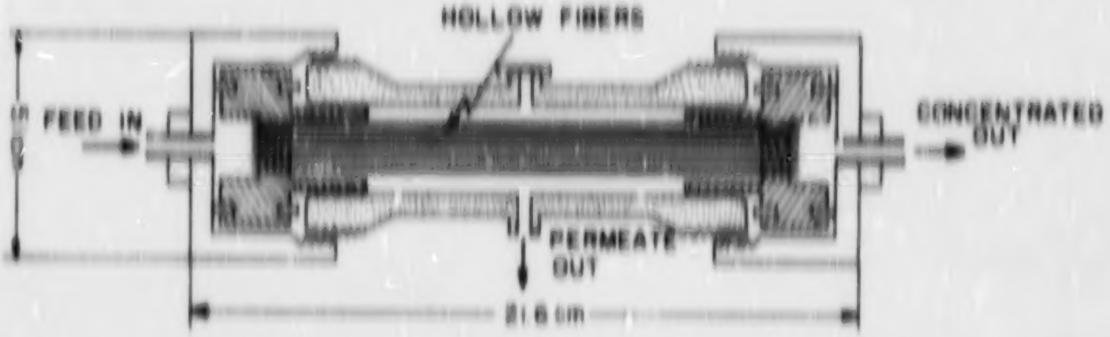
MATERIALS AND METHODS

Apparatus

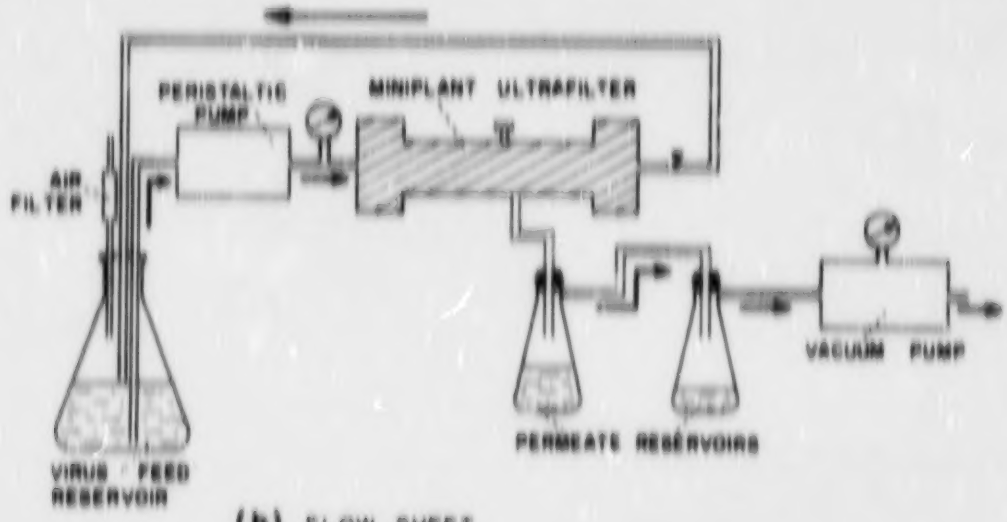
A commercially available hollow fiber membrane permeator (Bio-Fiber 80 Mini-plant from Bio-Rad Laboratories, Richmond, California, 94804, U.S.A.) was used. The dimensions of the unit and fibers with the flow sheet are presented in Fig. 1a. The hollow fiber membranes were made of cellulose acetate with a nominal surface area of 10,000 sq. cm. and a nominal molecular weight cut-off of 30,000 (Dow product designation C/HFU-10). The feed, seeded with polio-type 1 virus was pumped (Cole-Palmer Model 7015) through the core of the fibers at a linear flow rate of 9.1 and 3.16 ml s^{-1}, and continuously recycled. The permeate, which passed radially from the inside to the outside of the fibers was drawn into a buchner funnel by a vacuum pump (Millipore XX60 220 50) operating at 25 in Hg (0.836 atm). Thus, the driving force for permeation was about 1 atm, since a small positive pressure was also exerted on the feed side.

Cell Cultures and Medium

Two continuous lines of African green monkey kidney cells, Vero, (Flow Laboratory, Ltd., Scotland) and BGM (kindly supplied by Dr. G. Berg) were used. The cells were grown on tissue culture plastic plates (Nunc, Denmark). The medium, Eagle's minimum essential medium, contained 10 per cent foetal bovine serum and was incubated at 37°C in 90-95 per cent relative humidity and 5% CO_2 atmosphere.


HOLLOW FIBERS
FEED IN
CONCENTRATED OUT
PERMEATE OUT
21.6 cm

(a) HOLLOW FINE FIBERS


PERISTALTIC PUMP
MINIPLANT ULTRAFILTER
AIR FILTER
VACUUM PUMP
PERMEATE RESERVOIRS
VIRUS FEED RESERVOIR

(b) FLOW SHEET

Fig. 1. Detail of filter unit and system flow sheet

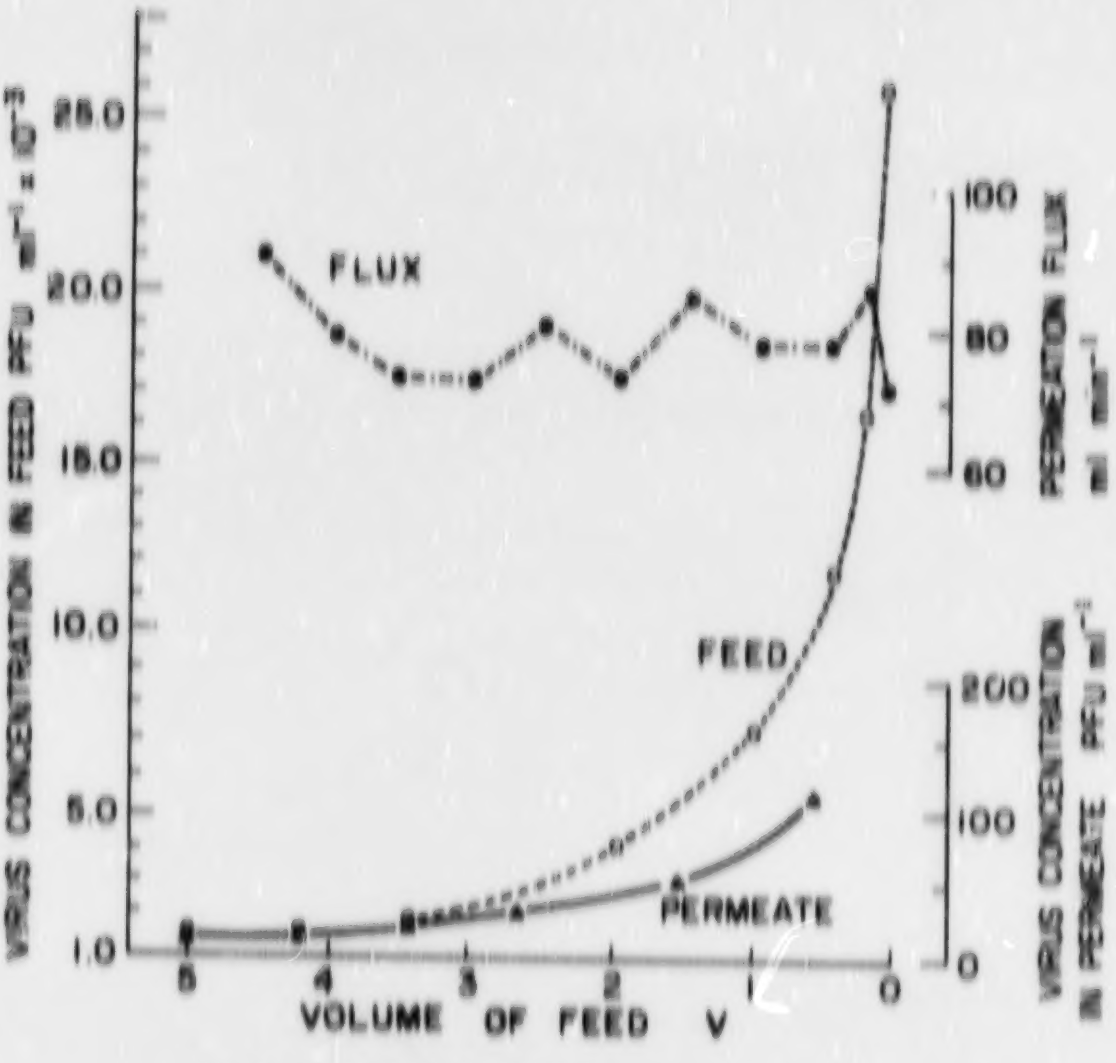


Fig. 7. Result of a typical virus concentration experiment

Table 10. RESULTS OF BACKWASH EXPERIMENTS

Exp. no.	Initial virus count^a pfu	Percent virus recovered in aliquots of permeate(%/ml) 1	2	3	4	5	Total percentage recovered (%)
14	7.415×10^6	34.5% 130 ml	1.7% 140 ml	–	–	–	36.2
15	2.335×10^6	53.8% 100 ml	12.7% 100 ml	–	–	–	65.5
16	2.265×10^6	26.9% 100 ml	1.9% 100 ml	–	–	–	28.8
17	7850	19.1% 50 ml	7.6% 95 ml	–	–	–	26.7
19	5550	22.3% 90 ml	5.6% 70 ml	–	–	–	27.9
20	5.165×10^6	28.2% 50 ml	10.7% 100 ml	0% 100 ml	–	–	38.7
21	9.165×10^6	6.55% 45 ml	4.0% 40 ml	1.8% 75 ml	0.8% 65 ml	0% 20 ml	13.2
22	235	54.5% 60 ml	0% 110 ml	–	–	–	54.5

^{a}In 5000 ml feed

Virus Assays

Virus assays were made by the plaque-forming unit (pfu) method, using an overlay medium consisting of Eagle's minimum essential medium, 2% foetal bovine serum and 1 per cent special agar-noble (Difco Labs., Detroit, Michigan, U.S.A.). Staining was done with Neutral Red.

Experimental Procedure

The required virus concentration (pfu ml^{-1}) was prepared in 5 ℓ of feed water and placed in the virus reservoir (see flow diagram in Fig. 1b). At time t = 0, both the peristaltic pump and the vacuum pump were switched on at a preset flow rate and vacuum setting, respectively. Ten ml samples for virus assays were taken at decreasing time intervals from both the feed and permeate reservoirs. The volume of permeate collected during each of these time intervals was measured and the average permeation flux (ml min^{-1}) was noted. The results were then plotted, as shown in Fig. 2, after each experiment.

RESULTS

The results for eleven experiments are presented in Table 10. The effect of each independent variable--flow direction (radially) through the hollow fiber, feed flow rate, initial virus concentration in the feed, cell type, and virus recovery using washing techniques--on the performance of the hollow fiber permeator is discussed below.

Radial Flow Direction

One of the major drawbacks in using hollow fiber membrane permeators for turbid waters in wastewater desalination is the high rate of flux decline(Belfort, 1974). This has been attributed to the poor hydrodynamic condition when the feed or turbid water is passed at high pressures (~40 atm) on the outside of the filaments. The best way these inherently weak fibers can withstand such high pressures is in compression with the feed solution on the outside.

Thus, it would seem logical from the outset, for the experiments discussed here, to operate the system with the feed solution on the inside rather than outside of the filaments. Firstly, because high pressures are not used here and secondly, well-controlled hydrodynamics as in tubular flow is essential so as to minimize virus loss. Another factor that should be considered is the structural characteristics of the membrane fiber wall. Depending on the method of preparation, fiber membranes can be made with a dense skin (or active layer) and an open spongy understructure, according to the original fabrication method of Loeb and Sourirajan[5]. Not all membranes have skins; while some may have a skin on either the bore surface or outer fiber surface. Skins are usually less than 1 per cent of the total membrane thickness. From Fig. 3, we see that the fibers used in these experiments are homogeneous and semi-dense throughout without a dense skin or open spongy understructure. The close-up SEM (Cambridge Scientific Instruments, Model 54-10) of the wall cross-section (Fig. 3b) displays a structure consisting of small pinholes and nodules very similar to

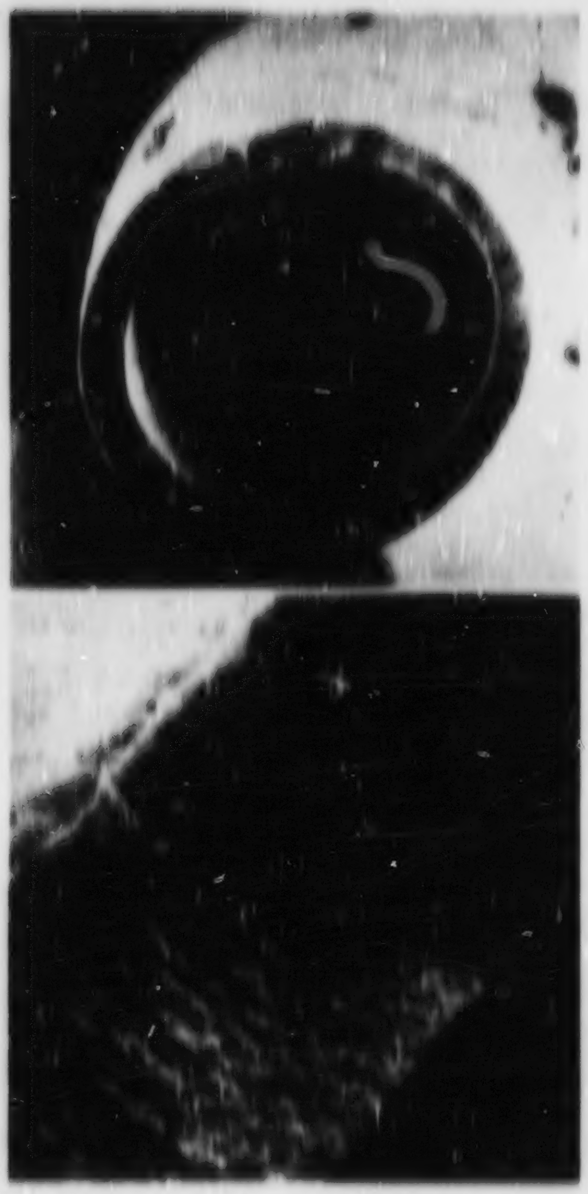


Fig. 3. Scanning electron micrographs of the cross-section of the hollow fiber membrane.
(a) Total fiber cross-section magnification: x 410, o.d.=170um; wall thickness=16um.
(b) Close-up of wall cross-section magnification: x 560, wall thickness=13um.

that described by Kesting[6]. The significance of this semi-dense structure is discussed later with regard to the backwashing experiments.

The average permeation rate for about 1 atm vacuum for radial flow from inside to outside, and outside to inside of the fiber was 94.8 and 86.2 ml min^{-1}, respectively. Since we wanted to maximize the permeation flux and minimize virus loss due to poor hydrodynamics, all experiments were run with the feed solution in the fiber capillaries and the vacuum on the outside of the fibers (see Fig. 1).

Feed Flow Rate

From the initial experiments (see Table 10, experiments 7, 11 and 12) we observed poor virus recovery efficiencies (< 40%) and suspected the well known phenomenon, concentration polarization, to be responsible for virus build-up and subsequent adsorption onto the membrane surface. Concentration polarization can be reduced by increasing the bulk flow rate (or Reynolds Number) inside the fiber thereby increasing the shear at the solution-membrane interface[7]. Experiment 14 was conducted at one third (3.16 ml s^{-1}) the usual feed operating flow rate of 9.1 ml s^{-1}. These flow rates correspond to Reynold's Numbers ($N_{Re} = dv/\eta$, where d is the internal fiber diameter, cm, v is the average internal flow velocity, cm s^{-1}, and η the kinematic viscosity of the liquid, cm^2 s^{-1}) of about 2.58 and 7.27, respectively. For tubular flow both are in the laminar regime.

From Fig. 4 and Table 11, we note that the concentration experiment can be adequately (certainly within the repeatability of the virus assay technique) described by the following relationship.[8]

$$C = AV^{-R}$$

where C = virus concentration in feed reservoir, PFU ml^{-1}

V = volume of the feed solution, ml

= $(V_0 - Qt)$

A = preexponent

= $C_0 V_0^R$

C_0 = initial C, pfu ml^{-1}

V_0 = initial V, ml

Q = permeation rate, ml min -1

t = time, min

R = rejection parameter

= $1 - C_p/C$

C_p = virus concentration in permeate, PFU ml^{-1}

The rejection parameter R is obtained from the slope of the linear plots in Fig.4 and describes the ability of a membrane to reject solute (virus). R varies from zero (poor rejecting membrane) to one (excellent rejecting membrane). From Table 11, of all the experiments listed only Experiment 12 had a significantly different slop R. The average coefficient of permeability L_p as presented in Table 10, however, is not significantly

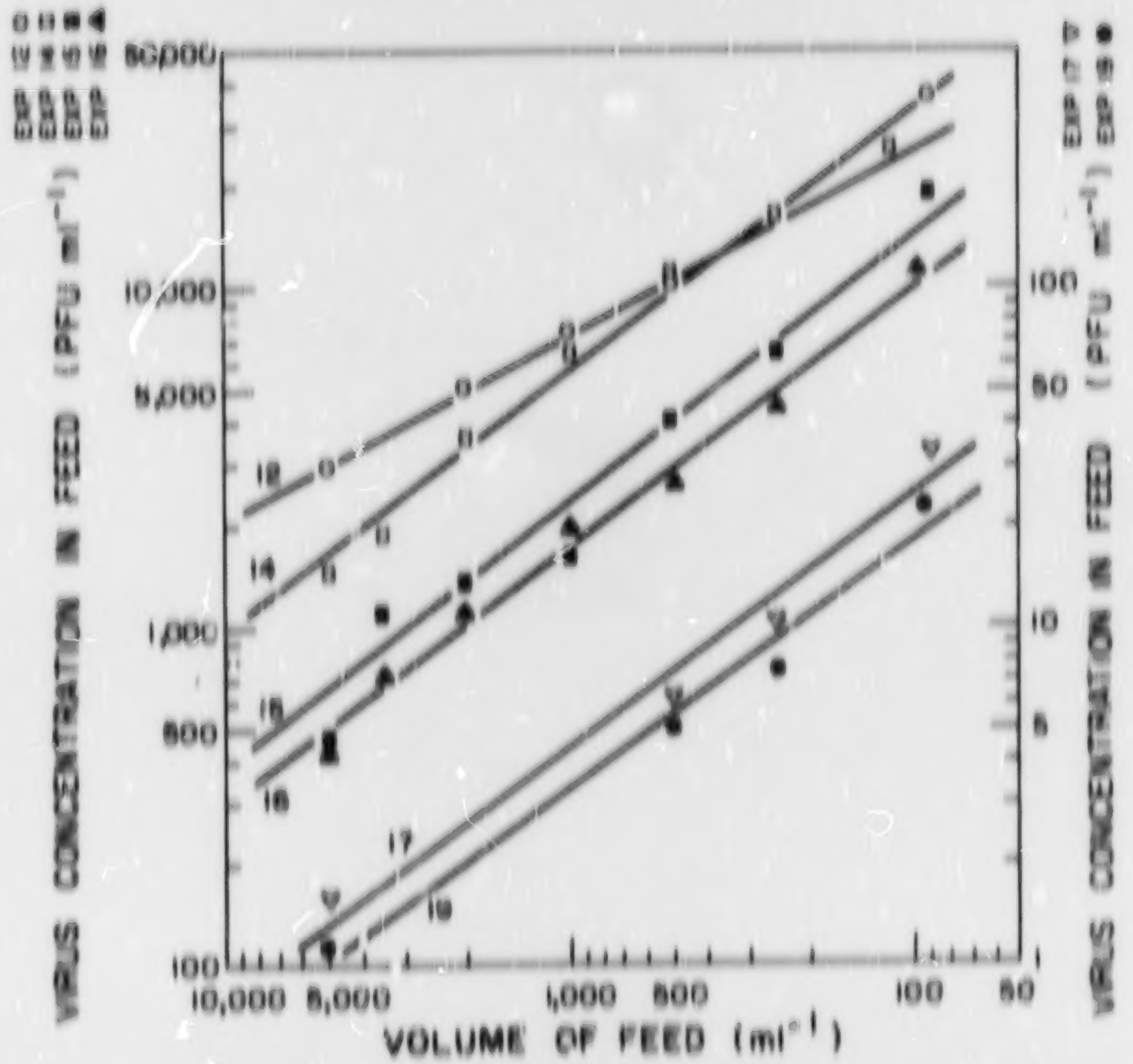


Fig. 4. Concentration of virus in feed versus volume of feed

Table 11. PARAMETRIC RESULTS FROM CURVE FITTING[a]

Exp. no.	A pfu ml^{-1}	Rejection R
12	303,000	0.54(1)
14	1,151,000	0.76(7)
15	515,200	0.78(3)
16	327,800	0.76(0)
17	775	0.70(0)
19	485	0.73(6)

[a] See Fig. 4

different for Experiment 12 than for the other experiments listed in Table 11. For Experiment 14 (slow flow rate), the value of L_p is on the low end of the range of values reported.

We thus conclude that for the feed flow rates 3.16 and 9.1 ml s^{-1}, no significant difference with respect to virus rejection was detectable. However, decreasing the average feed flow rate slightly reduced the permeation flux, indicating an increased resistance due possibly to surface adsorption. Moreover, the recovery of virus from washing experiments is of the same order of magnitude for the slow and fast feed rate experiments. (See the difference between the last two columns in Table 10 for, say Experiments 14 and 20). Thus, the large percentage of virus held-up in the fiber system must be due to some phenomenon other than only adsorption onto the fiber surface. A suggested explanation for this effect will be provided later in the discussion on backwashing.

Initial Virus Concentration and Cell Type

From Tables 10 and 11 and Fig. 4 , we note that for a range of three orders of magnitude of initial feed concentration (Experiments 14, 15, 16, 17 and 19) the R parameter is virtually identical. Experiment 12, however, has a feed concentration of 3000 pfu ml^{-1} and shows a 20 per cent drop in the rejection parameter R. Based on this result, all we can make is a preliminary statement to the effect that from 3000 pfu ml^{-1} and above the membrane rejection is probably adversely affected by the initial virus concentration. This concentration level is, however, far above that for sewage samples (40 pfu ml^{-1}) or secondary treated effluents (57 pfu ml^{-1}).[9]

Although it is known that the same virus concentration will plate about three times more pfu ml^{-1} for the BGM than the Vero cells, no significant difference is observed between the cells used in the assay technique for the concentration rate experiments presented here (see Experiments 15 and 16 in Fig. 4).

Backwashing experiments

From the first few experiments (see Experiments 7, 11 and 12 in Table 10) it became obvious that over 60 per cent of the virus was disappearing, either due to inactivation or adsorption on or within the membrane. The first possibility was evaluated by conducting the concentration experiment for 75 min without vacuum to see if continual recycle of the feed solution caused virus inactivation. No detectable inactivation or virus loss was observed. This experiment also indicated that the virus did not have a propensity to adsorb onto the inner fiber surface. This second possibility, virus adsorption onto the inner fiber surface, was also dismissed as a major cause of virus loss by the feed flow rate experiments. The third possibility, virus adsorption within the fiber wall, was tested by drawing a vacuum on the inside of the fibers and placing virus free water on the outside. Aliquots of water that permeated the fibers were collected and assayed for virus. The results are presented in Table 12.

Table 12. RESULTS OF VIRUS CONCENTRATION EXPERIMENTS

Exp. no.	Speed of feed[a]	Cells[b]	Feed concentration[c] (pfu ml^{-1})	Final volume of feed[d] (ml)	Duration (min)	Average permeability[e] L_p (ml cm^{-2} h^{-1} atm^{-1})	Virus recovery Without washing (%)	Virus recovery With washing (%)
7	F	?	667	130	61.5	5.70×10^{-1}	37.6	—
11	F	Vero	767	100	71.5	4.93×10^{-1}	32.6	—
12	F	BGM	3000	90	58.5	6.05×10^{-1}	34.2	—
14	S	BGM	1430	115	62.5	5.54×10^{-1}	56.3	92.5
15	F	BGM	467	90	63.0	5.62×10^{-1}	44.2	110.1
16	F	Vero	453	95	59.0	5.99×10^{-1}	49.3	78.1
17	F	BGM	1.57	90	58.5	7.00×10^{-1}	35.8	62.5
19	F	BGM	1.11	92	56.0	6.31×10^{-1}	54.0	87.9
20	F	BGM	1053	115	54.5	5.44×10^{-1}	51.1	88.8
21	F	BGM	1833	130	56.5	6.29×10^{-1}	64.6	77.8
22	F	BGM	0.94(?)	47	58.1	6.13×10^{-1}	28.5	78.67

[a] The feed stream was pumped either fast(F=8.1 ml s^{-1}) or slow (S=3.16 ml s^{-1})

[b] Viruses were assayed on either Vero or BGM cells

[c] Initial feed volume was 5l

[d] Final volume before washing

[e] To convert to (gpd ft^{-2}), multiply (ml cm^{-2} h^{-1}) by 5.8887. An important parameter is L_p, water permeability, which is obtained by dividing the average flux by the applied pressure difference (about 0.3333 atms here).

From Table 12, we see that large percentage of the missing virus can be recovered with relatively small volumes of wash water. Indeed, most of the wash-recovered virus is collected in the first and second aliquots (in about 150 ml). From the last column in Table 10, we note, however that from 10 to 20% of the original virus is still missing. It is possible that virus degradation and subsequent inactivation due to the forced movement into and out of the semi-dense membrane (see SEM in Fig. 3b) is responsible for this small virus loss.

The reason such a large amount of the original virus is held-up in the fiber wall could be explained by the inherent structure of the fiber itself. The lack of a dense skin which would prevent virus intrusion into the membrane and the existence of a homogeneous semi-dense wall, could explain this hold-up. Ideally, these experiments have indicated that a fiber with a dense skin located on the bore surface or inside of the fiber wall would serve to minimize virus intrusion and maximize the permeation or dehydration rate. Experiments using this system are at present underway in our laboratories.

CONCLUSIONS

1. The core-feed cellulose acetate hollow fiber unit was successfully tested as a potential virus concentrator for the first step of a two step sequential process to quantify virus counts in large volumes of water.

2. Dehydration ratios of greater than 50 in about 1 h and an average polio type 1 virus recovery of 84 per cent (average of all the experiments) were obtained.

3. Feed polio type 1 virus concentrations below 1500 pfu ml^{-1}, feed flow rate and cell type had no significant effect on the virus rejection. At 3000 pfu ml^{-1} however, the rejection was decreased by 20 per cent.

4. Linear plots were obtained for log (virus concentration) versus log (volume of feed). An average polio-1 virus rejection of 75 per cent was obtained with the fiber unit.

5. From 13 to 54 per cent of initial virus was recovered by backwashing. An average of 16 per cent of the virus was inactivated or damaged during the experiments due to intrusion of the virus into the semi-dense fiber membrane wall.

6. The method does not require adjustments of the pH of the water and there is no interference by organic matter or salinity.

REFERENCES

1. Bio-Rad Laboratories (Richmond, California 94804, U.S.A.) The New Hollow Fiber Technique. Bulletin 1004, 1972.

2. Belfort G. Pressure-driven membrane processes and wastewater renovation. In: Wastewater Renovation and Reuse, Shuval, H.I. (ed.). New York, Academic Press, 1975.

3. Sorber, C.A., J.F. Malina, and B.P. Sagik. Virus Rejection by Reverse Osmosis--Ultrafiltration Processes. Water Res. 6:1377-1388, 1972.

4. Sweet B.H., J.S. McHale, K.J. Hardy, F. Morton, J.K. Smith and E. Klein. Concentration of Virus from Water by Osmotic Ultrafiltration-I. Water Res. 5:823-829, 1971.

5. Sourirajan. Reverse Osmosis. London, Logos Press, 1970.

6. Kesting, R.E. Concerning the Microstructure of Dry-RO Membranes. J. Appl. Poly. Sci. 17:1771-1784, 1973.

7. Brian P.T.L. Desalination by Reverse Osmosis. In: Mass Transport in Reverse Osmosis, Merton, U. (ed.). Cambridge, Mass., MIT Press, 1966.

8. Deinzer, M., R. Melton and D. Mitchell. Trace Organic Contaminants in Drinking Water: Their Concentration by Reverse Osmosis, submitted to Water Res., 1974.

9. Buras, N. Recovery of Viruses from Waste-water and Effluent by the Direct Inoculation Method. Water Res. 8:19-22, 1974.

SECTION VII

EVALUATION OF GAUZE PAD METHOD TO RECOVER VIRUSES FROM WATER

INTRODUCTION

In 1948 Moore[1] described for the first time the use of gauze pads for the isolation of microbial pollutants from sewage. MacCallum et al[2] adapted this method for the detection of poliomyelitis virus in sewers. Melnick et al[3] were the first to point out that a gauze pad soaked in flowing sewage, yields a significantly higher percentage of positive tests for viruses than the grab sample method. However, the findings obtained, using the gauze pad, are qualitative and not quantitative, therefore some groups tried to evaluate gauze pad efficiency in the recovery of viruses from water. Coin et al[4] designed a gauze pad sampler for the quantitative measurement of viruses in water. Hoff et al[5] designed a modified sampler which was evaluated by Liu et al[6] for its efficiency in concentration of poliovirus from fresh and seawater and for some parameters affecting the efficiency, e.g. the pH, and calf serum.

The simplicity of the gauze pad method, as well as its high percentage of positive tests, prompted us in 1970 to undertake a study to quantitate this method. The aim of this study was to evaluate the gauze pad method for virus concentration from water and also to compare it to the grab method in field studies.

MATERIALS AND METHODS

Virus and Virus Assays

Attenuated poliovirus type I served as a model for laboratory studies. The virus was kindly supplied by the Ministry of Health's Virus Laboratory in Tel-Aviv. The stock virus was seeded on Vero cells, harvested and subdivided for storage at -70°C into volumes of 2 ml. Virus was assayed according to the plaque forming unit (pfu) method as described by Shuval et al in 1970.[7]

Cell Culture

1. A Vero cell line obtained from Flow Laboratories Ltd. Scotland, was grown and maintained in M-199 medium with the addition of 10% calf serum.
2. Primary monkey kidney cells, Rhesus obtained from the Ministry of Health's Virus Laboratory, Jaffe-Tel-Aviv, were trypsinized, grown and maintained in M-199 medium with the addition of 10% calf serum.

Antibiotics

The antibiotic stock solution contained 200,000 penicillin units, 200 mg of streptomycin, 5 mg kanamycin, and 4 mg neomycin per 1 ml H_2O.

Gauze Pad

Two kinds of sterile surgical gauze pads were used. For field studies, a pad weighing about 5 grams was folded into 16 layers and tied with a nylon cord to which a weight was attached. For laboratory experiments, a pad weighing about 6 grams was folded into 24 layers with a diameter of 10 cm.

Gauze Pad Sampler Apparatus

The gauze sampler is shown in Fig. 5. All parts of the sampler are made of stainless steel except for the rubber disc. The sampler contains: a. 5 liter stainless steel container, 23 cm diameter, 22 cm length; b. and c. 2 parts of the gauze holder, each of 0.5 liter capacity, diameter 10 cm; d. rubber disc; e. gauze pad; f. stainless steel screen, each of its pores having a 2 cm diameter.

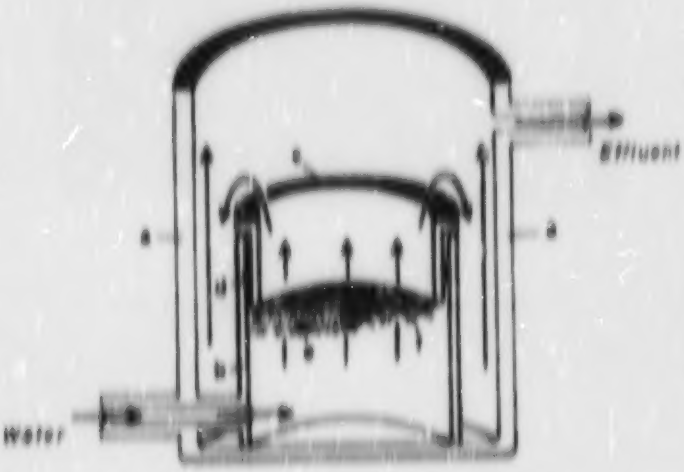


Fig. 5. Gauze sampler

The sampler in operation is shown in Fig. 6. The water from the tank (c) enters the gauze sampler (d) through an opening in the bottom. The water level rises slowly passes through the gauze pad (3), overflows and is discarded.

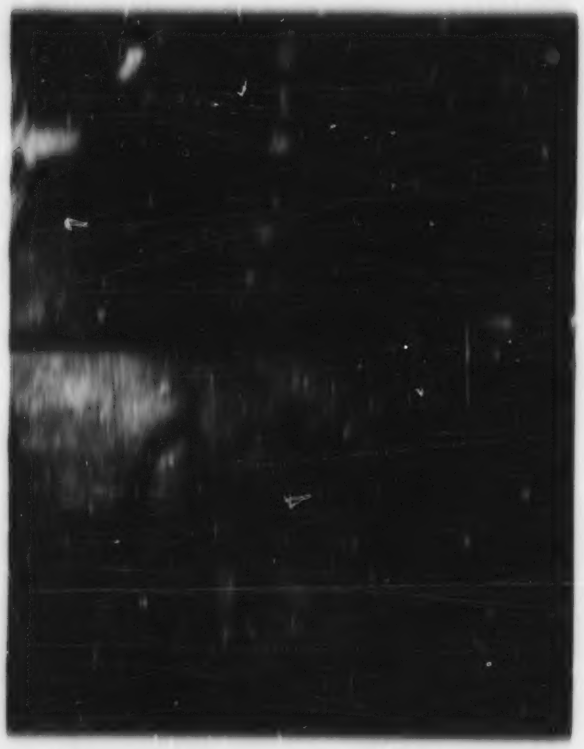


Fig. 6. Sampler in operation

PROCEDURES FOR SAMPLING AND ELUTION

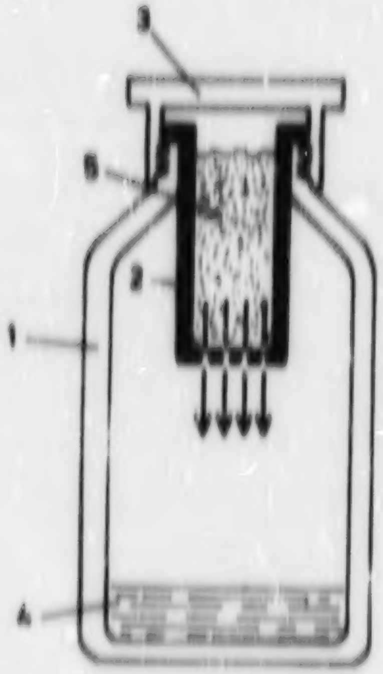
Field Study -

Two sampling methods were used and compared for concentration and detection of viruses from sewage: (1) Grab Sample Method and (2) Gauze Pad Method.

(1) Grab sample method - Twenty-nine daily samples of raw urban sewage were taken during the period of June 6 to July 12, 1970, and weekly samples were taken in the period from July 13 to September 27, 1970. Near the outlet of the sewage pipes, a composite grab sample of 3 liters sewage was collected for 60 minutes between 7:00 and 8:00 a.m. Every 10 minutes, 500 ml of sewage was collected and transferred to a sterile plastic vessel. The vessels were kept in an ice bucket, flown to the laboratory on the same day, and refrigerated at 4°C until the following day. Two liters of each sample were then concentrated by the phase separation method.[8] After concentration, the viruses were stored at -20°C until seeded on primary monkey kidney cells.

(2) Gauze pad method - During the above period from June to July, 29 gauze pads were each immersed in the sewage outlet mentioned previously, for 24 hours. In addition, 10 gauze pads were immersed at the same outlet during the second period from July to September for various periods of time: 3 pads for 3 days, 2 pads for 4 days and 5 pads for 7 days.

After the pad was removed from the sewage, it was transferred to a nylon bag and the latter was placed in a plastic bottle, cooled and conveyed to the laboratory as described. Several drops of a 1% solution of NaOH were added to the nylon bag containing the gauze to adjust the pH to 8.0. The liquid in the gauze was hand expressed. The expressed liquid was sterilely transferred to a glass cylinder. The gauze was expressed twice more, once after the addition of 10 ml of sterile distilled water, pH 8.0, and once after the addition of saline, pH 8.0. The 3 eluants were pooled and the gauze was transferred to a specially designed polypropylene insert with a perforated bottom, placed in a standard 250 ml centrifuge tube (Fig. 7) and centrifuged at 4000 x g for 10 minutes in a cooled centrifuge. Nearly all of the liquid content in the pad could thus be recovered. The volumes collected ranged from 50-80 ml. In order to sediment the bacteria, the liquid was recentrifuged for 30 minutes at 12,000 x g. After centrifugation, 1 ml of the stock antibiotic solution was added per 10 ml supernatant. The samples were kept at $-20^{0}C$ until seeded on primary monkey kidney cells.



1 = 250 ml plastic centrifuge bottle
2 = Polypropylene insert perforated bottom
3 = Screw cap
4 = The expressed liquid
5 = Gauze pad

Fig. 7. Device for extracting liquid for gauze pad

Laboratory Experiments -

For laboratory experiments, various volumes of dechlorinated tap water, pH 6.0, containing different virus concentrations, were filtered through the gauze sampler at a positive pressure of about 0.1 atmospheres. The rate of sample flow was 2.5 liters per minute. After filtration, the gauze pad was removed from the sampler with the aid of tweezers and transferred into a plastic bag. The pH was adjusted to 8.0 with a 1% NaOH solution. In some experiments, sufficient calf serum was added to attain 5% of the total volume of the fluids. The pad was then hand-pressed 5-7 times and centrifuged at 4000 x g for 10 minutes.

The dry pad was replaced in the plastic bag together with 20 ml saline, pH 8.0, to which, in some experiments, 5% calf serum was added. The processes of absorption, expression and centrifugation were repeated to obtain second, third, fourth and fifth eluates. One ml stock antiobiotic solution was added to every 10 ml of eluate. Each eluate was stored at -70°C until assayed.

RESULTS

Field Study

A Comparison of Virus Concentration by the Two Sampling Methods - Grab and pad - Table 13 presents the results of enterovirus concentration in raw sewage by daily grab samples together with the results of enterovirus concentration by 24 hour pad samples. Since the

Table 13. A COMPARISON OF ENTEROVIRUS CONCENTRATION BY TWO SAMPLING METHODS: GRAB (2 LITERS) AND PAD (24 HOURS)

No.	Grab pfu/2 L[a]	24 Hours Pad pfu/pad	Pad / Grab
1	2340	5414	2.31
2	3120	468	0.15
3	2666	7016	2.63
4	4680	15400	3.29
5	1680	5670	3.38
6	6360	11365	1.79
7	6280	16500	2.63
8	1480	12333	8.33
9	5560	10241	1.84
10	1180	5445	4.61
11	2200	8039	3.65
12	1760	7429	4.22
13	1800	6967	3.87
14	2040	4517	2.21
15	2600	6453	2.48
16	440	244	0.55
17	1140	9821	8.61
18	780	2053	2.63
19	2400	294	0.12
20	3600	948	0.26
21	2160	2195	1.02
22	1660	2020	1.22
23	1280	6641	5.19
24	1600	7084	4.43
25	1220	2955	2.42
26	1780	4437	2.49
27	2920	10240	3.51
28	4800	6622	1.7[illegible]
29	2620	8213	3.13
Average			2.82
S.D.			±2.08

[a] Average of grab samples on day of immersing the pad and day of removal

volume of the grab samples collected was always 2 liters, the concentration of viruses in 2 liters sewage sample was compared to the concentration in the liquid expressed from pads immersed in the same sewage outlet for 24 hours.

From Table 13 it can be seen that in 4 cases only (No. 2, 16, 19 & 20) the recovery of viruses using grab sampling method was higher than that in the pad samples and in 87% of the gauze samples, the virus recovery is higher than in the 2 liter grab samples. On the average, the number of viruses detected with the aid of the 24 hour gauze pad is about 3 times larger than the average number found in 2 liters of the grab sample at the moments of immersion and removal of the pad.

A Comparison Between the Concentration of Viruses in Gauze Pads Kept in Sewage for 24 Hours as Opposed to Those Kept 3, 4, and 7 Days at the Same Time -

Several pads were immersed in raw sewage for different periods of time: 3 pads for 3 days, 2 pads for 4 days and 5 for 7 days. For purposes of comparison, the average percentage of enteroviruses detected in gauze pads immersed in sewage for 24 hours was assumed to be 100%. The results of this comparison are summarized in Table 14.

From Table 14 it can be seen that in 9 out of 10 pads held for more than 24 hours, the percentage concentration of virus was equal to or less than that of pads immersed for 24 hours. In only one sample (7 days), was the percentage concentration twice that of the 24 hour sample.

The average percentage of virus concentration in pads kept for 3, 4 or 7 days ranged between 50-80%, i.e., less than the 24 hour average for the same period. It is worthwhile to mention that the amount of viruses in pads kept for 24 hours ranged on the average between 2511-6599 pfu/pad.

Table 14. THE AVERAGE PERCENTAGE OF ENTEROVIRUS DETECTED ON GAUZE PADS HELD IN SEWAGE FOR 1 DAY COMPARED WITH THE PERCENTAGE ON GAUZE PADS HELD FOR 3, 4, AND 7 DAYS

(1 day assumed as 100%)

No.	Average of 1 day %	3 days %	4 days %	7 days %
1	100.0	5.1	107.0	213.9
2	100.0	105.0	18.4	107.6
3	100.0	41.2	--	9.0
4	100.0	--	--	33.3
5	100.0	--	--	36.2
Average	100.0	50.4	62.7	80.0

A Comparison Between Three Gauze Pads Run in Parallel in Raw Sewage. Two sets of triplicate gauze pads were immersed for 24 hours in canals of flowing raw sewage and then similarly treated in the laboratory for virus detection. The results are presented in Table 15. The results show that there are no significant differences among the triplicates of each set.

Table 15. COMPARISON BETWEEN THE NUMBER OF VIRUSES DETECTED FROM 3 GAUZE PADS IMMERSED IN PARALLEL IN RAW SEWAGE

No.	pfu/PAD Pad I	 Pad II	 Pad III	Ave. pfu	S.D.
1	4575	6605	7138	6106	±1352
2	8772	8934	9504	9070	± 384

Laboratory Experiments

The field study has shown that the pad method is superior to the grab method. However, the gauze pad method is not a quantitative due to the fact that: the flow rate of the water passed through the gauze, the amount of virus adsorbed to the pad, the number passed right through it and the amount of unrecovered virus from the total adsorbed--are all unknown.

Because of the above and since it has not been possible to quantitate field results, we decided to test the pad method under controlled laboratory conditions in order to evaluate field results quantitatively.

The Effect of Repeated Elutions -

The effect of successive elutions on virus recovery from gauze pads immersed in tap water and flowing sewage was determined.

Tap water - Various volumes (700-51,500 ml) of dechlorinated tap water were filtered through the gauze in the sampler shown in Fig. 5. In some experiments each gauze was eluted 4 times (table 16); and in others, each gauze was eluted 5 times (Table 17).

From Table 16, it can be seen that the first eluate contained, on the average, 47% of the total virus obtained from gauze in 4 elutions. The second eluate contained, on the average, 25%; i.e., 72% is eluted after the first 2 elutions. Table 17 shows also on the average, 72% after the first 2 elutions. However, the average in the first eluate is 42%. The fourth eluate in Table 16 and the fifth eluate in Table 17 contained the least amount of virus.

Sewage [illegible] effect of repeated elutions with sewage was tested. Pads were immersed in flowing sewage for 24 hours. Each gauze was eluted four times. The results are presented in Table 18.

As shown in Table 18, the first eluate contained an average of 50% of the total virus obtained from the gauze in 4 elutions, whereas the second eluate contained an average of 25%, i.e. 75% is eluted after the first two elutions. It should be pointed out that Table 18 presents data for wild enteroviruses from sewage, whereas Tables 16 and 17 present those for seeded attenuated poliovirus 1.

Table 16. THE EFFECT OF SUCCESSIVE GAUZE PAD ELUTIONS ON POLIOVIRUS I ELUTION

(TAP WATER-4 ELUTIONS)[a]

Eluate	Exp. 1		Exp. 2		Exp. 3		Exp. 4		Average	
	pfu	%	pfu	%	pfu	%	pfu	%	%	S.D.
1st	73	67.6	122	57.9	2709	34.3	2250	26.7	46.6	±19.3
2nd	13	12.1	37	17.7	2736	34.7	3103	36.8	25.4	±12.3
3rd	18	16.2	42	20.0	1430	18.1	1511	17.9	18.1	± 1.6
4th	4	4.1	9	4.4	1017	12.9	1573	18.6	9.9	± 6.8
Total	108	100.0	210	100.0	7892	100.0	8437	100.0	100.0	

[a] In this table and in the subsequent ones, each elution is expressed as a percentage of the sum total virus (100%) obtained from the gauze in the sum total elutions.

Table 17. THE EFFECT OF SUCCESSIVE GAUZE PAD ELUTIONS ON POLIOVIRUS 1 ELUTION (TAP WATER – 5 ELUTIONS)

Eluate	Exp. 1		Exp. 2		Exp. 3		Exp. 4		Exp. 5		Exp. 6		Average	
	pfu	%	pfu	%	pfu	%	pfu	%	pfu	%	pfu	%	%	S.D.
1st	1486	25.1	4965	52.7	46400	33.2	73002	41.4	96000	54.4	83335	43.2	41.6	±11.3
2nd	2076	35.1	2508	26.6	44145	31.5	48776	28.0	44800	25.4	65400	33.4	30.1	± 3.8
3rd	812	13.7	976	10.4	22940	16.2	30101	17.0	21200	12.0	21332	11.0	13.5	± 3.0
4th	940	16.1	632	6.7	12600	9.0	11332	6.6	9000	5.1	17860	9.3	8.8	± 3.9
5th	612	10.3	342	3.6	14796	10.0	10003	5.8	5536	3.1	6019	3.1	6.0	± 3.4
Total	5915	100.0	9427	100.0	$1.4x10^5$	100.0	$1.7x10^5$	100.0	$1.8x10^5$	100.0	$1.9x10^5$	100.0	100.0	

Table 18. THE EFFECT OF SUCCESSIVE GAUZE PAD ELUTIONS ON ENTEROVIRUS RECOVERY

(Raw sewage)

Eluate	pfu	%	pfu	%	pfu	%	Average %	S.D.
1st	5250	59.8	4500	50.4	3825	40.2	50.1	±9.8
2nd	1611	18.4	2417	27.1	3125	32.9	26.1	±7.3
3rd	1244	14.2	1371	15.3	1915	20.2	16.6	±3.2
4th	667	7.6	646	7.2	639	6.7	7.2	±0.5
Total	8772	100.0	8934	100.0	9504	100.0	100.0	

The Effect of Calf Serum on Elution Recovery Efficiency of Viruses from Gauze Pads-

Since it has been found that the addition of serum to eluting fluid significantly affects recovery efficiency of virus from pads (Liu et al[5]), experiments were performed in order to compare the effect of calf serum (5%) added to a saline eluant. In each experiment, equal volumes of tap water, pH = 8.0, containing equal concentrations of attenuated poliovirus I were passed through separate gauzes. The two gauzes were treated as follows: one gauze was eluted 4 times with saline containing 5% calf serum, pH = 8.0, the other was similarly treated but without the addition of calf serum. The results are summarized in Table 19.

Table 19 shows that in 2 experiments (2 and 4), the addition of serum was slightly advantageous, whereas in 2 other experiments (1 and 3), saline was the better eluent. The fifth experiment shows no effect of the serum on elution. At any rate, after 2 elutions, with or without serum, about 75% of the total virus obtained from 4 elutions is released.

The Efficiency of Recovery of the Gauze Sampler for Viruses in Water-

Twenty experiments were carried out in order to determine the efficiency of the gauze pad sampler. This was done by comparing the concentration of viruses before passage through the gauze to that found on the gauze after filtration. The results of these experiments are summarized in Table 20.

Table 19. THE EFFECT OF CALF SERUM ON ELUTION OF POLIOVIRUS 1 FROM GAUZE PADS (TAP WATER)

	Exp. 1		Exp. 2		Exp. 3		Exp. 4		Exp. 5		Ave.	
Eluate	with C.S. %	with-out %	with C.S. %	with-out %	with C.S. %	with-out %	with C.S. %	with-out %	with C.S. %	with-out %	with C.S. %	with-out %
1st	57.9	67.6	54.7	27.6	26.7	34.3	56.1	36.9	44.6	43.9	48.0 ±13.9	42.1 ±15.4
2nd	17.7	12.1	27.6	39.2	36.8	34.7	25.2	35.1	34.5	30.6	29.6 ±7.5	30.3 ±10.6
3rd	20.0	16.2	10.7	15.3	17.9	18.1	12.4	18.0	11.4	18.5	14.6 ±4.2	17.2 ±1.4
4th	4.4	4.1	7.0	17.9	13.0	12.9	5.3	10.0	9.5	7.0	8.8 ±5.5	10.4 ±5.8
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Total virus on pad	1.6×10^2		7.2×10^3		8.2×10^3		1.5×10^5		1.7×10^5			

Table 20. THE RECOVERY OF THE GAUZE PAD SAMPLER FOR CONCENTRATION OF POLIOVIRUS I (TAP WATER)

		Before concentration		After concentration		Recovery
Exp. no.	Volume ml	Total pfu	pfu/ml	pfu/ml	Total pfu/pad	efficiency %
1	700	9.0×10^3	12.9	13.3	531	5.9
2	700	4.9×10^4	70.0	80.0	3.2×10^3	6.6
3	700	5.4×10^4	77.5	105.0	4.2×10^3	7.7
4	7500	9.7×10^4	12.9	22.9	917	1.0
5	7800	5.5×10^5	70.0	162.5	6.5×10^3	1.2
6	7800	7.0×10^5	77.5	185.0	7.4×10^3	1.1
7	9000	9.3×10^3	1.0	2.7	108	1.2
8	10000	2.8×10^3	1.0	5.3	211	2.2
9	10000	2.8×10^5	28.1	197.3	7.9×10^3	2.8
10	10000	4.0×10^5	41.7	210.9	8.4×10^3	2.1
11	19000	2.5×10^4	1.3	3.5	141	0.6
12	30000	1.1×10^6	38.1	147.9	5.9×10^3	0.5
13	30000	1.9×10^6	59.4	235.5	9.4×10^3	0.5
14	30000	1.1×10^7	350.0	4500.0	1.8×10^5	1.6
15	30000	1.3×10^7	446.7	3500.0	1.4×10^5	1.1
16	30000	2.4×10^7	840.0	4750.0	1.9×10^5	0.8
17	30000	2.9×10^7	966.7	4250.0	1.7×10^5	0.6
18	51500	6.6×10^5	12.9	45.0	1.8×10^3	0.3
19	51500	3.6×10^6	70.0	450.0	1.8×10^4	0.5
20	51500	4.0×10^6	77.5	725.0	2.9×10^4	0.7

From Table 20, it can be seen that:

(1) The recovery efficiency of viruses by the gauze pad is low: 0.3 - 7.7%

(2) The recovery efficiency of the viruses is a reciprocal function of the sample volume and not of the total number of viruses passed through the gauze: the larger the volume, the lower the efficiency (an efficiency of 7.7% for 700 ml versus 0.3% for 51,500 ml).

(3) On the other hand, a comparison between the amount of virus per ml (pfu/ml) found in the expressed gauze fluid and the amount of virus per ml (pfu/ml) in the control sample (before concentration) shows that in each experiment the amount of viruses after concentration in the expressed gauze fluid is higher than that of the control. (Compare columns 4 and 5 in Table 20).

The Effect of Sample Volume on Virus Recovery Efficiency- In order to verify the recovery efficiency of viruses as a reciprocal function of the sample volume, controlled experiments were carried out as follows:

Attenuated poliovirus I at a concentration of 12.9 pfu/ml was added to 60 liters of dechlorinated tap water. The 60 liters were then divided into 3 aliquots: 700, 7,800 and 51,800 ml, each of which was passed through a separate gauze. Each gauze was subsequently uniformly eluted 4 times.

Table 21. THE EFFECT OF SAMPLE VOLUME ON POLIOVIRUS 1 RECOVERY FROM WATER

	Experiment no. 1				Experiment no. 2				Experiment no. 3				Ave. of 3 exp.			
	Virus concentration pfu/ml				Virus concentration pfu/ml				Virus concentration pfu/ml				Virus concentration pfu/ml			
Volume ml	In control	In gauze eluate	Virus in gauze / virus in control	% recovery	In control	In gauze eluate	Virus in gauze / virus in control	% recovery	In control	In gauze eluate	Virus in gauze / virus in control	% recovery	In control	In gauze eluate	Virus in gauze / virus in control	% recovery
700	12.9	13.7	1.0	5.9	70.0	80.0	1.1	6.6	77.5	105.0	1.4	7.7	53.5 ±35.3	66.2 ±47.4	1.2 ±0.2	6.7 ±0.9
7000	12.9	22.9	1.8	1.0	70.0	162.5	2.3	1.2	77.5	185.0	2.4	1.2	53.5 ±35.3	123.5 ±87.8	2.2 ±0.3	1.1 ±0.1
37850	12.9	45.5	3.5	0.3	70.0	480.0	6.9	0.5	77.5	725.0	9.4	0.7	53.5 ±35.3	416.8 ±344.0	6.6 ±3.0	0.5 ±0.2

In a second and third set of experiments, the virus concentration under similar conditions was 70.0 and 77.5 pfu/ml respectively. The results of the 3 sets of experiments are summarized in Table 21.

Table 21 shows the sharp decrease in recovery efficiency relative to the sample volume in each experiment: average recovery efficiency of 6.7% for 700 ml and 0.5% for 51.5 L. The concentration factor of the gauze is obtained by dividing the pfu/ml of gauze fluid after filtration by the pfu/ml of the control fluid before filtration. This factor increases in direct proportion to the sample volume: the factor is 1.2 for 700 ml versus 6.6 for 51,500 ml.

DISCUSSION

This study is divided into two parts: (a) field study for comparison of two sampling methods, pad and grab samples; and (b) laboratory experiments for evaluation of the gauze pad method for concentration of viruses from water.

For the second part, we designed a flow-through gauze sampler (Figs. 5 and 6) in order to quantitate the gauze pad method. A new method involving centrifugation for the expression of gauze liquid was used (Fig. 7). The centrifugation method enabled the expression of 95% or more of the liquid absorbed by the gauze.

The findings of this study show that the recovery efficiency for concentration of viruses by gauze pads from tap water is very low and dependent on sample volumes (0.5% for 51 liters versus 7% for 700 ml). These findings are in agreement with those of Liu et al.[6] On the other hand, we found that the concentration factor of the pad is dependent on the virus concentration in volumes up to 30 liters: volumes greater than 30 liters show no effect (1.2 for 700 ml and 6.4 for 30 or 51 liters).

The addition of calf serum to the wash fluid did not significantly affect elution of viruses from pads immersed in tap water, see Table 19. Liu et al[6] found that the addition of calf serum also enhances elution from tap water pads, dependent on pH. On the average, about 60% of the viruses are eluted in the first wash as compared to about 70% in the first two washes, see Tables 16 and 17.

According to our results (Table 13), we find that the concentration of virus in 2 liters of sample by the grab method is 3 times lower, on the average, than that of liquid expressed from the 24 hour pad. Obviously, different results would have been obtained if we had used gauze of different size or used different volumes of grab samples. If we compare equal volumes of gauze and grab, i.e., the concentration of virus in 1 ml of liquid expressed from a gauze pad kept in sewage for 24 hours compared with the average concentration in 1 ml of grab sample on the day of immersion and the day of pad removal, we find that the gauze liquid contains 90 times more virus than the number found in the sewage in which it was immersed.

In our field work, two sample methods were employed: grab sample and gauze pad. Composite samples were taken by the grab method and concentrated by the phase separation method (Shuval et al[8]). This method, though it is quantitative, has been shown to be somewhat selective in its ability to concentrate all enteroviruses (Grindrod & Cliver[9]). On the other hand, the gauze pad method though advantageous in sampling for a continuous and long period of time (24 hours and more), is limited by being qualitiative, since we don't know the flow rate, how much virus is adsorbed to the pad, and how much recovered. In addition, we, as well as other groups (Hoff et al,[4] Liu et al[6]) find that only a very low percentage (about 1%) of the entire enterovirus population can be concentrated by this method. In spite of these limitations, various investigators have found the gauze pad method to be superior to the grab method in the detection of viruses.[3] It may be that the limitations are overcome by the large volume of sewage which passes through and by the pad's ability to pick up clumps of viruses, excreted by individual carriers, which enter the sewage intermittantly. Grab samples may completely miss such peaks in virus concentration.

In addition, the concentration of viruses in sewage during 24 hours may differ from that found at the moment of grab sampling. However, it was found that the concentration of virus in the gauze eluent is always higher than the concentration of virus in the sewage surrounding the gauze, i.e., 3 to 410 times higher, and this means that the viral recovery of the pad in sewage is quite effective despite the low efficiency.

It was found that there is no advantage in immersing the gauze pads for more than 24 hours, see Table 14. Lund and Hedstrom[10] found that pads immersed for 24 contained more viruses than those maintained for 48 hours.

A comparison between the number of viruses detected in gauze pads immersed in parallel in raw sewage reveals only slight differences (Table 15). Lund and Hedstrom[10] found it advantageous to maintain two gauzes simultaneously as they discerned small differences in the concentration as well as different strains in the two gauzes.

In spite of the limitation of the gauze pad method (low recovery efficiency and qualitative results) we think that this method is useful and may be of practical value in epidemiological studies for early discovery, or epidemiologic intelligence work on viral outbreaks even before clinical signs can be diagnosed, since virus excretion into sewage precedes the clinical syndromes.

REFERENCES

1. Moore, B. The detection of Paratyphois in towns by means of sewage examination. Mon. Bull. Minist. Hlth. Publ. Hlth. Lab. Serv. (directed by Med. Res. Council), 7:241-248, 1948.

2. MacCallum, F.O., W.C. Cockburn, E.H.R. Smithard, and S.L. Wright. The Use of Gauze Swabs for the Detection of Poliomyelitis Virus in Sewers. Second Int. Poliomyelitis Conf., Lippincott, Phila. p. 434-437, 1952.

3. Melnick, J.L., J. Emmons, M. Opton, and J.H. Coffey. Coxsackie Viruses from Sewage. Methodology Including an Evaluation of the Grab Sample and Gauze Pad Collection Procedures. Amer. Jour. Hygiene 59:185-195, 1954.

4. Coin, L., M.L. Menetrier, J. Labonde, and M.C. Hannoun. Modern Microbiological and Virological Aspects of Water Pollution. Direction de l'Hygiene Sociale, Paris, 1961.

5. Hoff, J.C., R.D. Lee, and R.D. Becker. Evaluation of Method for Concentration of Microorganisms in Water. Water Hygiene Division, 1971, p.37.

6. Liu, O.C., D.A. Brashear, H.R. Seraichekas, J.A. Barnick and T.G. Metcalf. Virus in Water: A Preliminary Study on a Flow-Through Gauze Sampler for Recovering Virus from Waters. Appl. Microbiol. 21:405-410, 1971.

7. Shuval, H.I., A. Thompson, B. Fattal, S. Cymbalista and Y. Wiener. Natural Virus Inactivation Processes in Sea Water. In: Proceedings of the National Specialty Conf. on Disinfection, New York, American Society of Civil Engineers, pp 429-452, 1970.

8. Shuval, H.I., B. Fattal, S. Cymbalista, and N. Goldblum. The Phase-Separation Method for the Concentration and Detection of Viruses in Water. Water Research 3:225-240, 1969.

9. Grindrod, J. and D.O. Cliver. Limitation of the Polymer Two Phase System for Detection of Viruses. Archive fur die gesamte Virusforschung 28:337-347, 1969.

10. Lund, E. and C.E. Hedstrom. A Study on Sampling and Isolation Method for the Detection of Viruses in Sewage. Water Res., 3:823-832, 1967.

SECTION VIII

A RAPID FLUORESCENT ANTIBODY METHOD FOR QUANTITATIVE ISOLATION OF VIRUSES FROM WATER

INTRODUCTION

The accepted techniques for virus isolation involve inoculation of tissue cultures followed by incubation. Virus is then demonstrated by the appearance of cytopathic effect or plaques in the tissue cultures. The entire process continues for 3-7 days or sometimes even longer, depending on the method.

It is obvious that a method for testing potable water sources requiring, at least, 3 days does not provide an adequate degree of protection, especially when contaminated river water is distributed to large populations within hours after passing through a treatment plant. Such tests should be as rapid as possible, and the assay should be completed before the water is released to the distributing system.

Rapid qualitative methods for demonstration of enteroviruses based on the fluorescent antibody (FA) technique have already been described.[1,2,3,4] In this study efforts were made to develop it one step further, thus affording a rapid quantitative determination of enteric viruses in water. As a model, poliovirus type I was chosen.

MATERIALS AND METHODS

Virus Stock

Poliovirus type I (Brunhilda) was used throughout this study. The virus was grown in Vero cells (Flow Laboratories, Scotland), accumulated and concentrated by the phase separation method.[5,6] Vials containing 0.5 ml of the concentrated viruses were stored at -80°C. Before each test a sample was defrosted and diluted according to the experimental requirements. The virus was assayed on BGM cells,[7] as described elsewhere.[8]

Antiserum

Rabbits were injected in the footpad with 1.0 ml emulsion containing equal volumes of concentrated poliovirus I (2.3×10^{10} pfu/ml) and complete Freund's adjuvant (Difco Laboratories). Second, third and fourth identical injections were administered after 2, 6 and 10 weeks, respectively. Fourteen days after the last injection, blood was withdrawn from the ear veins and the serum separated.

Purification and Concentration of Gammaglobulin

This was carried out as described elsewhere.[9]

Labeling of Gammaglobulin

Labeling was performed as described previously[10] with the exception of the temperatures (about 20°C) and the stirring which was continued for 4 hrs. Isomer I of fluoresceinisothiocyanate (FITC: BDH, England) was used throughout this study.

Vero Cell Powder

Powder was prepared by acetone-drying from saline-suspended cells in the same manner as with liver powder.[11]

Removal of Non-specific Staining

The labeled gamma globulins were adsorbed by the Vero cell powder to remove non-specific staining in the following manner: 100 mg powder were suspended in saline and centrifuged at 1,000 x g for 15 min; 5 ml labeled gamma globulin were added to the sediment and mixed. This suspension was incubated at 37°C for one hour, followed by centrifugation at 40,000 x g for 30 min. The supernatant was considered adsorbed serum. Additional adsorptions were sometimes necessary until complete removal of non-specific staining was achieved. A solution of rhodamine-labeled bovine albumin (Microbiological Associates) was added to the adsorbed serum (final dilution 1:40) to obtain a clear differentiation between positive and negative reactions.

Micro Tissue Culture for FA Staining

A suspension was prepared containing 2-3 x 10^5/ml BGM cells in M-199 medium with Hank's salts (Flow Laboratories, Scotland), 20% fetal bovine serum and antibiotic solution (penicillin 200 units, streptomycin 200 μg, kanamycin 5 μg, neomycin 4 μg per milliliter final concentration). This mixture was inoculated with virus according to the requirements of each experiment. Five drops (0.02 ml/drop) were put on a standard microscope slide, several slides per experiment. The slides were then placed into specially designed vessels containing small volumes of water to prevent dehydration, and incubated in a CO_2 incubator at 37°C. Unless otherwise stated, the length of incubation was 18-20 hrs. After incubation, the slides were rinsed with 0.15 M phosphate buffer, pH 7.2, followed by three washings in acetone.

Fluorescent Antibody Staining

The direct method was used,[11] with the staining continuing for one hour. The stained preparations were examined under a Zeiss WL Research microscope with fluorescent attachment.

Concentration of Viruses from Water and Their Inoculation into Tissue Cultures

Viruses were concentrated from the water by filtration through a cellulose nitrate membrane filter (pore size 0.45 μ; Sartorius Co., Germany). For volumes of 5 liters, the technique of Rao and Labzoffsky[12] was utilized. The membrane diameter was 47 mm. The adsorbed virus was eluted from the membrane with 7 ml 3% beef extract.[13] For the testing of 40 L volumes, concentration of the viruses was carried out in two stages, according to Sobsy et al,[14] with the following modifications: the pH of the water sample was adjusted to 3.0 and filtered through a cellulose nitrate membrane filter (diameter 142 mm, pore size 0.45 μ). To elute the adsorbed virus, 100 ml glycine buffer (0.2 M, pH 11.5) was filtered through the membrane. The pH of the eluate was adjusted to 3.0 with 1 M HCl, and the suspension was again filtered (filter diameter 47 mm). The adsorbed virus was eluted with 7 ml 3% beef extract.[13]

One ml sterile M-199 medium (concentrated 10 fold), 2 ml fetal bovine serum and 0.1 ml antibiotic mixture (200,000 U penicillin, 200,000 ug streptomycin, 5,000 ug kanamycin, 4,000 ug neomycin per ml) were added to each of the 7 ml concentrate obtained in both procedures. BGM cell (0.1 ml, 4-6 x 10^6) were added to 2 ml of the concentrate.

Micro tissue cultures were prepared on microscope slides with this virus/cell suspension as described above. The remainder of the virus was used for inoculation of the tissue cultures on plates.[8]

RESULTS

Growth of Poliovirus in Micro Tissue Cultures

To determine the optimal time required for FA staining of the cultures, infected micro tissue cultures were prepared and incubated. The first slide was removed after incubation of one hour, washed, fixed and stained. The second slide was treated in the same manner after incubation of two hours. This procedure was followed for 24 consecutive hours. All slides were examined microscopically. Incubation of one hour showed the cells to be attached to the slide; nearly all were still spherical and separated one from the other. All cells stained reddish-brown, the color of the rhodamine bovine albumine used as the counter stain.

Four hours after incubation, the cells appeared as in mature tissue cultures; they were flattened and attached to one another, giving the impression of monolayers. The cells were still stained with the counter stain.

Individual cells stained with the green fluorescence of the fluorescent antibodies started to appear after 6-7 hours of incubation, and after 9 hours a maximum of individual stained cells was reached. They appeared as isolated green spheres, surrounded by the reddish brown stained cells (Fig. 8).

After an incubation period of 16 hours the positive cells started to appear in foci of 5-30 cells, looking like clusters of green fluorescent spheres (Fig. 9). The number of positive cells increased in each infected foci until, after 22-24 hours, the clusters had grown into small plaques, each comprising scores of cells.

Quantitative Estimation of Poliovirus with FA Staining

The foregoing experiments indicated that the growth process of poliovirus in micro tissue cultures, as revealed by the FA staining, can be divided into two stages: stage 1, reaching its peak at 9 hours, during which positive cells remain single entities, and stage 2, with the peak at about 16-24 hours, when the positive cells form clusters. It is reasonable to assume that both the single positive cells appearing at 9 hours and the clusters at 16-24 hours, each represent one plaque forming unit of the original suspension. To test this contention, a number of experiments were carried out.

Fig. 8. BGM Cells infected with poliovirus type I, stained with fluorescent antibodies; 9 hours after infection. Two positive (white) single cells are clearly seen (x 100)

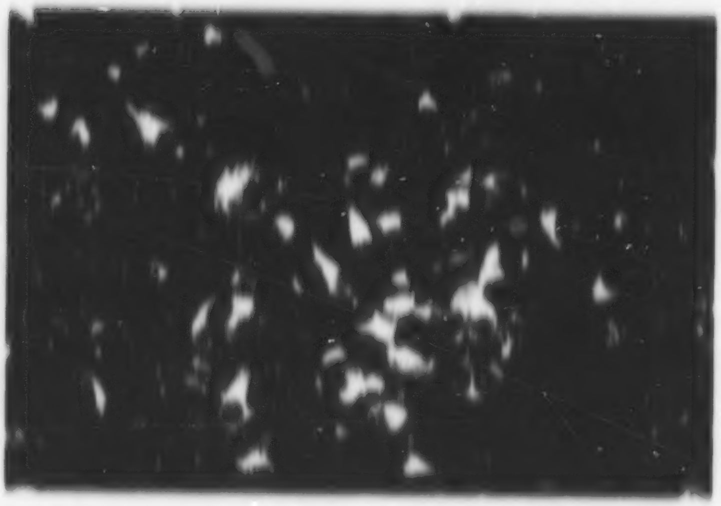


Fig. 9. BGM cells infected with poliovirus type I, stained with fluorescent antibodies; 18 hours after infection. The cluster of positive (white) cells is clearly defined (x 100)

Micro tissue cultures, infected with serial 10-fold dilutions of poliovirus, were prepared as described in methods and divided into two batches. One batch was incubated for 9 hours and the other for 20 hours. The FA stained preparations were examined thoroughly under the microscope. The single positive cells (9 hrs) and the clusters of positive cells (20 hrs) were counted. Simultaneously, the same poliovirus stock was titrated on plates as a control (Table 22). There is agreement between the FA preparations incubated for 20 hours and the controls. The 9-hour incubate, however, shows less satisfactory results, with a virus titer far below that of the controls. Another important factor with the 20-hour incubate was the readily discernible clusters, which should enable any technician with minimal experience to read results with ease. The single stained cells (9 hours), on the other hand, are often difficult to recognize or to distinguish from non-specific fluorescence. In view of these obstacles, 9-hour incubates were discontinued and the results described below concern only samples taken at 16-24 hours.

Table 23 compares the poliovirus titers of the FA stained cells and the plaque assay method. The results of the plaques were read two days after those of the FA. The virus titers in both methods are nearly identical.

Quantitative Detection of Poliovirus in Water

After it was established that the above procedure enables a rapid and quantitative assay of poliovirus in controlled experiments, it was

Table 22. TITERS OF POLIOVIRUS 1 OBTAINED WITH THE FLUORESCENT ANTIBODY METHOD AFTER 9 AND 20 HOUR INCUBATION, AND WITH THE PLAQUE COUNT METHOD ON PLATES

Exp. no.	Incubation period (hours)	Dilution of viruses sample	No. of positive cells or cell groups per drop	Calculated virus titer	Virus titer on plates
1	20	10^{-4}	3, 4, 7, 3	2.1×10^{7}	3.4×10^{7}
		10^{-5}	2, 1, 1, 2	7.5×10^{7}	
	9	10^{-2}	165, 190, 164	8.7×10^{6}	
		10^{-3}	23, 21, 18	1.0×10^{7}	
2	20	10^{-4}	2, 3, 1, 2	1.8×10^{7}	3.0×10^{7}
		10^{-5}	1, 1, 0, 1	3.8×10^{7}	
	9	10^{-1}	78, 61, 67	3.4×10^{5}	
		10^{-2}	8, 5, 2	2.6×10^{5}	
3	20	10^{-4}	4, 3, 4, 2	1.6×10^{7}	2.6×10^{7}
		10^{-5}	2, 2, 1, 0	6.3×10^{7}	
	9	10^{-2}	58, 44, 53, 26	2.1×10^{6}	
		10^{-3}	6, 4, 3, 4	2.1×10^{6}	

Table 23. TITERS OF POLIOVIRUS TYPE I OBTAINED WITH THE FLUORESCENT ANTIBODY TECHNIQUE AND WITH THE PLAQUE COUNT METHOD

Exp. no.	Virus titer	
	Plaque count	FA technique
1	3.0×10^7	2.6×10^7
2	2.7×10^7	6.2×10^6
3	1.5×10^7	1.1×10^7
4	2.0×10^7	1.2×10^7
5	1.1×10^7	1.2×10^7
6	2.2×10^7	1.3×10^7
7	1.8×10^7	1.5×10^7
8	1.4×10^7	1.4×10^7

decided to investigate its practical application--that is, the quantitative isolation of the virus from large volumes of water. Two accepted methods for the concentration of viruses from water were utilized in this study: one designed for volumes of 5 liters, and the other for larger bodies of water, in our case, a volume of 40 liters. Tap water was contaminated with 10-1,000 pfu poliovirus 1, according to the requirements of the experiment, concentrated and titrated simultaneously on micro cultures and plates. Tables 24 and 25 summarize the results of experiments with the 5- and 40-liter samples, giving good correlation between the two methods. Because of contamination, no results could be obtained in three experiments with the plaque assay, a factor which did not come into play with the FA method (Table 25).

DISCUSSION

The main reason for virus monitoring of water is to protect the population from health hazards. It would be desirable if the quality of the water be known before it reaches the consumer. Since impoundment of vast quantities of water for long periods is undesirable, rapid results of water tests are imperative.

Standard bacteriological tests require 24 hours, but even this relatively brief period should be shortened for most water distribution systems. Virological tests which take days, and sometimes weeks, are, of course, even less efficient in providing early warning of contamination. The reason for this time lag is not inherent in the

Table 24. COMPARISON OF THE FLUORESCENT ANTIBODY TECHNIQUE WITH THE PLAQUE COUNT METHOD FOR QUANTITATIVE EVALUATION OF VIRUSES IN 5 LITERS OF WATER

Exp. no.	pfu recovered	
	FA counts	Plaque counts
1	800	920
2	800	624
3	110	942
4	90	140
5	100	153
6	100	167
7	100	133
8	28	37
9	55	35
10	55	66
11	24	56
12	20	61
13	26	44
14	32	61

Table 25. COMPARISON OF THE FLUORESCENT ANTIBODY TECHNIQUE WITH THE PLAQUE COUNT METHOD FOR THE QUANTITATIVE EVALUATION OF VIRUSES IN 40 LITERS OF WATER

Exp. no.	pfu recovered FA counts	 Plaque counts
1	22	33
2	36	8
3	3	7
4	7	contaminated
5	21	contaminated
6	14	contaminated
7	42	6
8	8	13
9	8	10

concentration methods. Although some of these methods are slow, as for example, the phase separation method which needs 24-48 hours,[5,6] most concentration methods require 1-6 hours only. On the other hand, the time needed for the isolation of virus is far more extended. The usual techniques involve inoculation of tissue cultures followed by incubation. Viruses are then demonstrated by the appearance of the cytopathic effect (CPE) or plaques in the tissue cultures. The entire process lasts from 3 to 7 days or longer, depending on the particular method. In our laboratory, the plaque assay is used, which enables quantitative evaluation of the virus 3-5 days after sampling. It is possible to shorten the time needed for the plaque assay, but the danger of incomplete plaque development then exists, which is expressed by too low a number of plaques or by false negatives.

The main objective of the present study was the development of a quantitative method for the isolation of viruses from water which would be at least as rapid as the current bacteriological methods.

Viruses enter drinking water sources by way of domestic sewage. In the latter, various types of viruses are present [15] most of which belong to the enterovirus groups, including the following sub-groups: polio, coxsackie and echo viruses. They are mostly found in sewage and their concentration ranges from 400-2,000 pfu/l,[15] sometimes reaching over 10,000 pfu/l. Of the entire enterovirus group, only the three polio types should always be present in domestic sewage of urban areas in developed countries. The reason is the wide-spread routine administration of live poliovirus vaccine to infants. The viruses multiply in the intestines and are excreted with the feces for several weeks after the initial administration. Their number in the feces may reach 10^7 pfu/gm.[16] The presence of other types of enteroviruses in sewage depends on the degree of their distribution amongst the population at a given time, and this of course fluctuates considerably. This fact should be borne in mind when water is tested for the presence of viruses. Moreover, a single system that could be used to isolate all the different types of enteroviruses does not exist. Most of the coxsackie A virus types do not multiply in tissue cultures but require suckling mice. Polio- and echovirus, on the other hand, grow in tissue cultures. Also, the length of time required for the development of the cytopathic effect varies for each virus type. For example, CPE of poliovirus is displayed in 3-5 days, while that of reoviruses appears considerably later. The isolation of the different virus types found in water requires a wide range of techniques and systems, a fact which makes routine practical use too complicated. In bacterial examinations, only one type of bacterium--coliforms--is taken as being representative for other enteric bacteria present in the feces, thus becoming the indicator for bacterial fecal pollution. It would therefore be logical to select a viral indicator, the poliovirus being the most suitable candidate. Such an indicator for viral pollution of water could simplify the technique since it is based on a single system. Furthermore, it would also significantly shorten the identification period.

However, there are certain limitations regarding the use of poliovirus as viral indicator. Unlike E. coli, they do not necessarily comprise the majority of viral population in sewage, and at times are not present at all (see Section IX of this report). Therefore, negative results obtained with tests based on polioviruses only, would not be sufficient proof for the absence of other dangerous viruses in water. On the other hand, the lack of a rapid virus test slows down the implementation of routine viral examination of potable water. The use of the three polioviruses as indicators is suggested for a rapid examination of water as part of a complete and comprehensive virological test that will include all possible viruses in water. Thus, a rapid and preliminary answer may be obtained as to the presence of viruses in water designated for human consumption. With this objective in mind, polioviruses were selected for the present study, with poliovirus I as model.

The process of the development of the CPE is slow and includes the following stages: infection of susceptible cells, multiplication and liberation of the progeny, upon which the cycle starts again. In the case of polioviruses, each cycle lasts for 6-9 hrs[17] at 37°C. Several such cycles are needed before the CPE can be detected visually; in other words, the results are detectable only after a few days. A reagent that would enable identification of the virus in the tissue culture, before the CPE becomes visible, would therefore allow for a much shortened test time. Fluorescent antibodies could here fulfill the role of such a reagent.

Final identification of viruses is currently carried out either by the use of specific antibodies which neutralize the appearance of CPE in infected tissue cultures or by fluorescent antibodies. The latter stain the cells containing viral antigens, thus enabling identifcation of viruses in the cell considerably before the visible CPE. It is noteworthy that poliovirus antigens were already demonstrated approximately 6 hours post infection and reached their maximal concentration 3 hours thereafter. Thus, by using fluorescent antibodies it is theoretically possible to determine the presence or absence of poliovirus in tissue cultures 6-9 hours after infection. The FA technique was suggested as a method for typing polioviruses in 1959[5] and it was later established as a reliable technique.[1,2,4] The value of this technique for the identification of poliovirus is beyond dispute.

The FA method requires a special microscope, not suitable for tissue cultures grown in bottles or on plates. A method of micro tissue cultures on microscope slides was therefore developed. The cells were inoculated with the virus while still in suspension and measured drops (0.02 ml) were placed on a slide thus making quantitative determination of the virus possible.

BGM cells,[7] used routinely in our laboratory for virus isolation from water, were employed throughout this study. It is reasonable that other types of cells may be suitable here, although the number of cells necessary to obtain a monolayer within a short period of time should then first be determined. The number of $2\text{-}3 \times 10^6$ cells/ml was chosen with BGM cells after trial and error. This number may not apply, however, to other cell types. Using BGM cells in the micro tissue culture method resulted in a monolayer after 4 hours. Twenty-four hours after the start of the experiment (the time needed for completion of the test) the cells still formed a monolayer.

Positive cells, stained by the FA technique, were already observed after incubation of 9 hours, but the results were not yet sufficiently quantitative. Furthermore, the finding and identifying of positive cells at a time when they are still scarce, enhanced the danger of 'false positive' or 'false negative' identification. On the other hand, after incubation of 18-24 hrs, the cell culture was at the end of the second virus growth cycle e.g., the progeny of each primarily infected cell had, in turn, infected the neighboring cells. Viewing these FA stained preparations, under the microscope demonstrated groups of positively stained cells. It is extremely simple to identify such cell groups, and even a relatively inexperienced person could easily recognize them. The results after 18-24 hrs incubation are almost identical to those obtained by the plaque count technique, with the difference that the latter method requires two additional days.

The rapid method could be utilized for various virological procedures and is not necessarily limited to water testing. Since our motivation was the virological examination of water, the proposed method was tried in combination with various techniques for concentration of viruses from water. Two accepted techniques, based on membrane filtration[12,14] were chosen for this purpose. They are simple to perform and have a high concentration factor of 1,000-10,000.

A 40-liter volume was concentrated to 7 ml of fluid. But even this small volume required scores of slides with the micro tissue culture system. Ten slides resulted in the demonstration of approximately 10 pfu/40 liters, which was the upper limit of sensitivity. Techniques with a higher concentration factor would enhance the sensitivity of the rapid method and simplify the procedure. For practical routine application, 0.5 ml final concentrate would be most suitable.

The rapid method and the accepted plaque technique, using concentrated water samples, yielded nearly identical numbers of viruses. Furthermore, apart from the fact that the rapid method showed results 2-3 days before the plaque technique, plates with the latter were often contaminated with bacteria, thus prohibiting reading of results. The incubation period in the rapid method was too short to allow contamination.

The objective of the study was to develop a rapid method for the isolation and identifcation of viruses from water. A time interval of 18-24 hours should satisfy most requirements for water distribution. It should be pointed out that, in fact, this rapid method allows for the demonstration of viruses in the water sample after only 6-9 hours of incubation. However, the possibility of 'false negatives' or 'false positives' has than to be taken into account, probably due to the impurities in the reagents used in the FA technique. Improving the quality of the reagents should overcome these obstacles and may enable virus identification in water within 9 hours or less.

REFERENCES

1. Brown, G.C. Fluorescent Antibody Techniques for the Diagnosis of Enteric Infections. Arch. Ges. Virusforsch. 13:30-34, 1963.

2. Hatch, M.H., S.S. Kalter and G.W. Ajello. Identification of Poliovirus Isolates with Fluorescent Antibody. Proc. Soc. Exp. Biol. Med. 107:1-4, 1961.

3. Kalter, S.S., M.H. Hatch, and G.W. Ajello. The Laboratory Diagnosis of Poliomyelitis with Fluorescent Antibodies. Bacteriol. Proc. p. 89-90, 1959.

4. Riggs, J.L., and G.C. Brown. Application of Direct and Indirect Immunofluorescence for Identification of Enteroviruses and Titrating Their Antibodies. Proc. Soc. Exp. Biol. Med. 110:833-837, 1962.

5. Shuval, H.I., S. Cymbalista, B. Fattal and N. Goldblum. Concentration of Enteric Viruses in Water by Hydro Extraction and Two Phase Separation. In: Transmission of Viruses by the Water Route, Berg, G., (ed.). New York, Interscience Publ., 1967. p. 45-55.

6. Shuval, H.I., B. Fattal, S. Cymbalista, and N. Goldblum. The Phase Separation Method for the Concentration and Detection of Viruses in Water. Water Res. 3:225-240, 1969.

7. Barron, A.L., C. Olshevsky, and M.M. Cohen. Characteristic of the B.G.M. Line of Cells from African Green Monkey Kidney. Arch. Ges. Virusforsch. 32:389-392, 1970.

8. Shuval, H.I., A. Thompson, B. Fattal, S. Cymbalista, and Y. Wiener. Natural Virus Inactivation Processes in Seawater. J. San. Eng. Div. ASCE 97:587-600, 1971.

9. Katzenelson, E., and H. Bernkopf. Studies of the P.L.T. Agents with the Aid of the Agar Immunodiffusion Technique. Amer. J. Ophthal. 63:1483-1487, 1967.

10. Katzenelson, E., and H. Bernkopf. Serologic Differentiation of Trachoma Strains and other Agents of the P.L.T. Group with the Aid of the Direct Fluorescent Antibody Method. J. Immunol. 94:467-474, 1965.

11. Nairn, R.C. Fluorescent Protein Tracing. 3rd Ed. London, E. and S. Livingstone, Ltd., 1969.

12. Rao, N.U., and N.A. Labzoffsky. A Simple Method for the Detection of Low Concentrations of Viruses in Large Volumes of Water by the Membrane Filter Technique. Can. J. Microbiol. 15:399-403, 1969.

13. Berg, G., D.R. Dahling, and D. Berman. Recovery of Small Quantities of Viruses from Clean Waters on Cellulose Nitrate Membrane Filters. Appl. Microbiol., 22:608-614, 1971.

14. Sobsey, M.D., C. Wallis, M. Henderson, and J.L. Melnick. Concentration of Enteroviruses from Large Volumes of Water. Appl. Microbiol. 26:529-534, 1973.

15. Shuval, H.I. Detection and Control of Enteroviruses in the Water Enteroviruses in the Water Environment Development. In: Water Quality Research. Ann Arbor-London, Ann Arbor Humphrey Science Publ., 1970. p. 47-71.

16. Sabin, A.B. Recent Studies and Field Tests with a Live Attenuated Polio Virus Vaccine. First International Conf. on Live Polio Viruses Vaccines. Pan American Sanitary Bureau, 1959. p. 14-33.

17. Lwoff, A., R. Dulbecco, M. Vogt, and M. Lwoff. Kinetics of the Release of Poliomyelitis Virus from Single Cells. Virology. 1:128-139, 1955.

SECTION IX

VIRUS TYPES IN ISRAEL SEWAGE

INTRODUCTION

Poliovirus and other enteroviruses are present in sewage during epidemic and nonepidemic periods (Ramos Alvarez et al).[1] In Israel where oral Sabin vaccination is administered routinely it is to be expected that poliovirus can be isolated regularly from sewage. In the period 1968-1970, we isolated and typed enterovirus from sewage of several communities in Israel in order to gain information of the general frequency of enterovirus types which were detected by the methods we were studying.

MATERIALS AND METHODS

Virus Assay

The sewage samples were collected by grab and gauze pad methods. Grab samples were concentrated by the phase separation method (Shuval et al),[2] and assayed for enteroviruses on primary monkey kidney monolayers by the plaque forming method (pfu). In the case of the gauze pad method, the liquid was sterilely expressed from the pad and assayed.

Cell Culture

Primary monkey kidney cells, Rhesus or Vervet, obtained from the Ministry of Health Virus Laboratory, Jaffa-Tel-Aviv, were trypsinized and maintained in M-199 medium with the addition of 10% calf serum.

Virus Typing

Two methods were used:

1. Picked Virus - A number of plaques from positive samples were transferred to individual tubes which were frozen and kept at -20^0C for identification. Virus typing procedures were similar to those used in routine clinical virus typing work (Lennette et al).[3] For the purpose of this study, viruses were classified in gross categories only. They were either identified as one of the three types of poliovirus (P_1, P_2, P_3) or, when found negative in neutralization tests against poliovirus antisera but producing paralysis when injected in suckling mice, they were classified as Coxsackie virus type B or Echovirus type 9. Strains found negative with polio antisera as well as in suckling mice, but exhibiting a cytopathogenic effect (CPE), were considered as possible strains of Echovirus (ECHO) or other unidentified enteroviruses.

Preparation of Antipolio Serum - Antiserum against poliovirus types 1, 2 and 3 was prepared by intramuscularly injecting a rabbit with 1 ml of a solution composed of 0.5 ml poliovirus (titer 10^9 pfu/ml) and 0.5 ml complete Freund's adjuvant (Difco Laboratories). The two components of the suspension were mixed just prior to injection.

Two booster doses of the same composition were injected at intervals of 14 days. Blood samples were withdrawn from the ear 7-10 days after each booster in order to determine the titer. When the titer was high, larger amounts of serum were withdrawn directly from the heart with a

50 ml syringe. The serum was separated from the samples, inactivated at 56°C for 30 minutes and subsequently stored at -20°C until further use.

2. Neutralization Test for Determination of Poliovirus in Sewage

A 0.15 ml volume of sewage sample, diluted to contain not more than 20 pfu of virus, was mixed with 0.15 ml rabbit serum containing antibodies against poliovirus types 1, 2 and 3, and diluted to 4 times the concentration necessary for neutralization of the amount of virus present. The control contained the same diluted sewage as well as an equal volume of M-199. After incubation at 36°C for 60 minutes, 0.3 ml of the mixture was seeded on a plated monolayer of monkey kidney. The results are expressed as pfu/ml. The amount of poliovirus in a 0.15 ml sample is the difference between the amount of virus neutralized in the sewage sample and the amount of virus in the control sample. The neutralization test was used for sewage grab samples concentrated by phase separation[2] as well as for sewage expressed from gauze pads immersed in sewage for 24 hours.

RESULTS

Virus Types Picked from Sewage

The results of virus strains picked from sewage at different places in Israel are shown in Table 26. During 1968-1970, 489 plaques were isolated. Seventy-four percent of all strains proved to be polioviruses: 25% polio 1, 12% polio 2 and 37% polio 3. Thirteen percent of the poliovirus strains showed a strong CPE when incubated at 40°C. The remaining 87% grew at 37°C only. The latter are considered

Table 26. VIRUS TYPES PICKED FROM SEWAGE AT DIFFERENT PLACES AND COMMUNITIES IN ISRAEL

Virus	KIRYAT SHEMONEH Raw Sewage		TIBERIAS Raw Sewage		Intake		Effluent		JERUSALEM Raw Sewage		BET SHEMESH & RAMLEH		[illegible]		Total	
Type	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Polio 1	20	19.0	15	16.7	23	31.0	53	32.3	11	28.2	1	14.3	1	10.0	124	25.3
Polio 2	7	6.7	19	21.1	7	9.5	18	11.0	2	5.1	1	14.3	3	30.0	57	11.7
Polio 3	36	34.3	34	37.8	29	39.2	68	41.5	11	28.2	0	0	3	30.0	181	37.0
Cox. B or Echo 9	13	12.4	11	12.2	7	9.5	10	6.1	2	5.1	1	14.3	3	30.0	47	9.6
Echo or Other	29	27.6	11	12.2	8	10.8	15	9.1	13	33.4	4	57.1	0	0	80	16.4
TOTAL	105	100.0	90	100.0	74	100.0	164	100.0	39	100.0	7	100.0	10	100.0	489	100.0

attenuated poliovirus vaccine strains. Ten percent of the strains were Coxsackie Type B or Echo 9 and 16% were Echovirus or other virus strains.

Polio and Non-Polio Viruses Tested by Neutralization

During the period 11 June 1970 through July 1970, 52 samples were taken from the main sewage pipeline of Kiriat Shemoneh, a settlement in northern Israel. Twenty-six grab samples and 26 gauze pads, which were held for 24 hours, were taken daily. These samples were tested for polio and non-poliovirus by the neutralization test. The average results are in Table 27.

Table 27. POLIO AND NON-POLIOVIRUS IN GRAB AND GAUZE PAD SAMPLES IN KIRIAT SHEMONEH SEWAGE BY NEUTRALIZATION TEST

No.	Sampling method	Ave. no. of enterovirus	Ave. no. of non-polio	% Poliovirus
26	Grab	7364 pfu/L	5111 pfu/L	36.9
S.D.		±9559	±8440	±19.9
26	Gauze Pad	17543 pfu/pad	11097 pfu/pad	37.0
S.D.		±17197	±12247	±20.1

From Table 27 it can be seen that the amount of the total non-poliovirus in grab as well as in gauze pad samples was higher than that of the total amount of poliovirus. An average of 37% of the daily grab and gauze pad samples was identified as poliovirus. These findings are lower than those found in Table 26. In Table 26, 60% of the picked plaques of Kiriat Shemoneh sewage was identified as poliovirus.

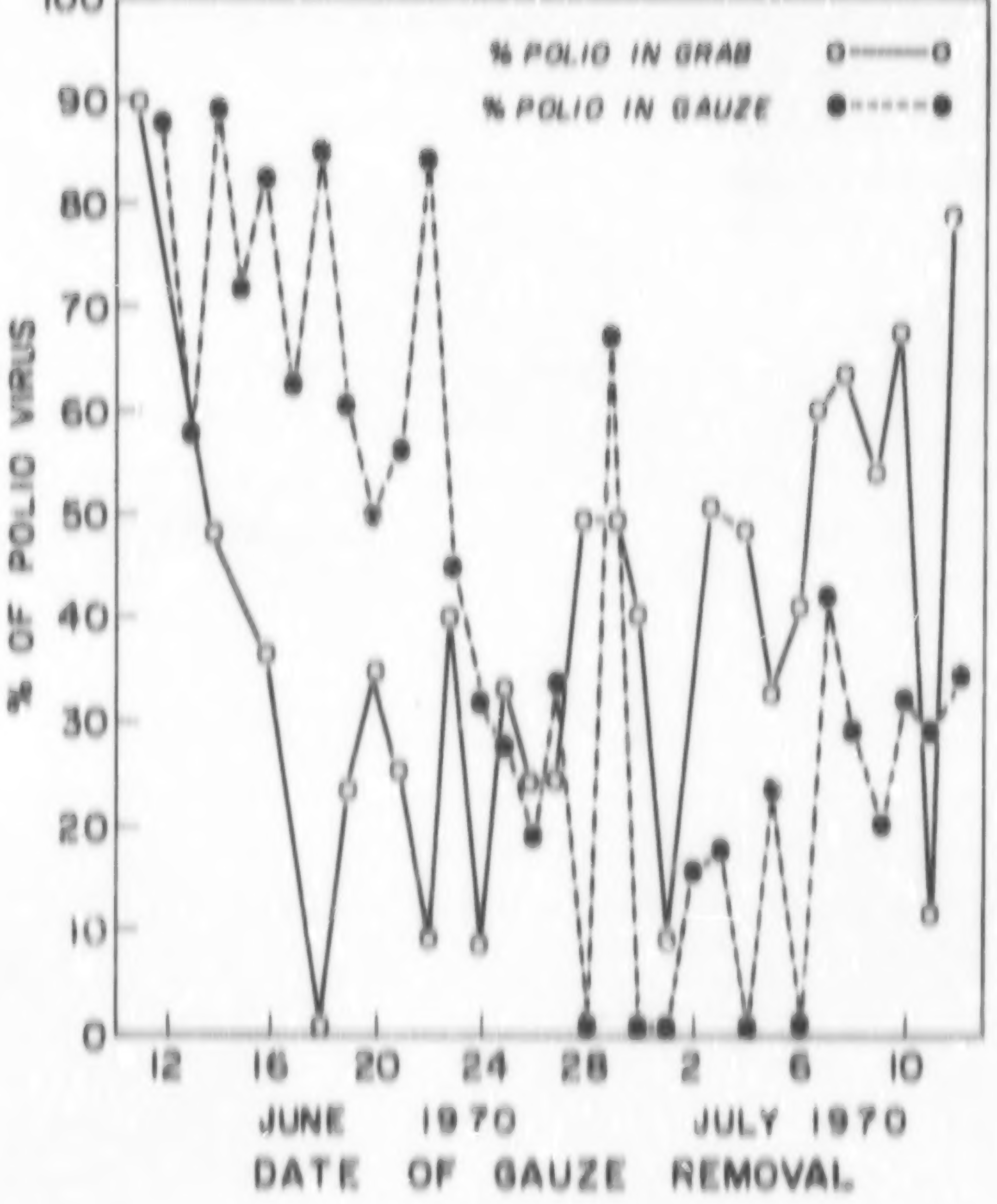


Fig. 10. The % of poliovirus in daily grab sampling vs. daily gauze pad in sewage by neutralization test

In Figure 10 we compare poliovirus in daily grab samples with gauze pad samples kept for 24 hours on the same day and from the same sewage. From this figure it can be seen that the percentage of the amount of poliovirus from daily gauze pads is not clearly correlated to that of the daily grab sampling. However, the average percentage of poliovirus found in 26 gauze pads and 26 grab samples was identical (37%).

DISCUSSION

Isolation and identification of enteroviruses in sewage of a particular community is very important from an epidemological point of view because it depicts the general situation of enteroviruses in that population.[4-9] They also can serve as a forewarning of an approaching enterovirus epidemic or of a carrier of the same.[10, 11]

In this study we isolated and identified enteroviruses in sewage from various communities in Israel. Our findings show that the percentage of poliovirus found by neutralization tests as well as by picked plaques is higher than that found by other researchers. Palfi[12] found 19% poliovirus from 317 isolated enteroviruses and Horbowska et al[13] found 22% polioviruses from 139 enteroviruses.

In Figure 10, it can be seen that in using the gauze pad sampling or grab sampling, there are daily fluctuations in poliovirus levels, and that in almost every sample checked, poliovirus was detected, in the picked plaques as well as in neutralization tests.

These findings seem to be reasonable in israel sewage where Sabin polio vaccinations are administered routinely to infants, four times within their first year.

It is difficult to reach a significant conclusion from these results, because of the variance of enterovirus concentration in sewage due to the change of season,[14] polio vaccinations administered within a short period of time,[15] or to enteroviral outbreak epidemic.[6] It is worthwhile to emphasize that our field and laboratory methods were selective for poliovirus. The concentration method,[16,17] the tissue culture cells, as well as the short incubation time are not able to detect certain enteroviruses, such as Reoviruses, Adenoviruses or Rhinoviruses,[3] and this can explain the high percentage of polioviruses that we found in the sewage. Regular virus monitoring of sewage can give a reliable picture of enteroviruses in sewage as well as of the prevalence of enteroviral diseases in the population.

REFERENCES

1. Ramos-Alvarez, M. and A.B. Sabin. Intestinal Viral Flora of Healthy Children Demonstrated by Monkey Kidney Tissue Culture. Amer. J. Public Health. 46:295, 1956.

2. Shuval, H.I., B. Fattal, S. Cymbalista and N. Golublum. The Phase-Separation Method for the Concentration and Detection of Viruses in Water. Water Res. 3:225-240, 1969.

3. Lennette, E.H. and N. Schmidt. Diagnostic Procedures for Viral and Rickettsial Infections. American Public Health Assoc. Inc., New York, 4th Edition, 1969.

4. Horstmann, D.M., J. Emmons, L. Gimpel, T. Subrahmanyan and J.T. Riordan. Enterovirus Surveillance Following a Community-wide Oral Poliovirus Vaccination Program: A Seven-Year Study. Amer. J. Epidemiol. 97(3):173-186, 1973.

5. Pittler, H., W. Hopken, and K.W. Knocke. Enteroviruses and Adenoviruses in Sewage: Demonstration and Epidemiological Study 1963-1965. Z BAKT Orig.(Germany). 204:33-48, 1967.

6. Wiley, J.S., T.D.Y. Chin, C.R. Gravelle and S. Robinson. Enteroviruses in Sewage During a Poliomyelitis Epidemic. In: Transmission of viruses by the Water Route, Berg, G. (ed.) New York, Wiley and Sons, 1967. p. 168.

7. Wilterdink, J.B., H.T. Welland, and J.D. Verlinde. A Longitudinal Study on the Significance of Examining Sewage for the Presence of Polioviruses in the Population. Arch. Ges. Virusforsch. 32:82-90, 1970.

8. Tarabcak, M., I. Kratochvil, and A. Milosovicova. Effect of Vaccination with Live Poliovirus Vaccine on the Circulation of Enteroviruses in the Population. J. Hyg. Epidemiol. Microbiol. Immun. 15(7):258-266, 1971.

9. Wilterdink, T.B., H.T. Weiland and T.D. Verlinde. A Longitudinal Study on the Significance of Examining Sewage for the Presence of Polioviruses in the Population. Arch. Ges. Virusforsch. 32:82-90, 1970.

10. Kuwert, L. P.G. Hohler, and C.A. Primavera. Intratypic Differentiation of Poliovirus Strains Isolated from Sewage: Consequences for the Practice of Oral Vaccinasion. ZBL BAKT Orig. (Germany). 215:16-25, 1970.

11. Nelson, D.B., R. Circo, and A.S. Evans. Strategic Viral Surveillance of Sewage during and FOllowing an Oral Poliovirus Vaccine Campaign. Am. Jour. of Epidemiology. 86:641-652, 1967.

12. Palfi, A.B. Virus Content of Sewage in Different Seasons in Hungary. Acta Microbiol. Acad. Sci. (Hungary). 18(4):231-237, 1971.

13. Horbowska, H., H. Wielopolska, H. Krolak. Virologic Studies on Sewage in Warsaw in the Years 1966-1971. Przegl Epidemiol. (Poland). 27(3):103-108, 1973.

14. Melnick, J.L., J. Emmons, J.H. Coffey, and H. Schoof. Seasonal Distribution of Coxsackie Viruses in Urban Sewage and Flies. Am. J. hyg. 59:164-184, 1954.

15. Zdrazilek, J., K. Zacek, J. Chvapil, V. Mikesova, L. Pokorna, V. Tomanova, J. Tranc, and J. Vrabkova. Virological Surveys for Enteroviruses in Prague at the End of 1960 and in 1969. CS Epidemiol. Microbiol. Immun. (Czechoslovakia). 20(3):67-72, 1971.

16. Grindrod J. and D.O. Cliver. Limitation of the Polymer Two Phase System for Detection of Viruses. Archiv. Fur Die Gesamte Virusforschung. 28:337-347, 1969.

17. Duff, M.F. Isolation of Ether Resistant Enteroviruses from Sewage: Methodology. Appl. Microbiol. 19(1):120-127, 1970.

PART B
INACTIVATION OF VIRUSES IN WATER

SECTION X
THE CHEMISTRY OF OZONE AS A DISINFECTANT

INTRODUCTION

Ozone has been used for the disinfection of water supplies since the beginning of the century when it was applied to the treatment of water for the City of Paris[1] and also at Nice, France.[2] At present, there are nearly 1000 installations in operation, mainly in Europe, but also including 20 in Canada where the largest is operating on drinking water supplied to the City of Quebec, treating flow rates up to 60 mgd.[3] Thus, while ozone is known to be an even more powerful disinfectant than chlorine[4] very little is known of the chemistry of ozone as a disinfectant. In contrast, thorough studies have been carried out on the chemistry of the halogens in water and the disinfection efficiencies of the different species present in the solution.[5] It is the purpose of this section to review the chemistry of ozone in water as it is known and to consider the potential disinfection efficiencies of ozone and its probable dissociation species in water.

DISSOCIATION OF OZONE IN WATER

Ozone is known to be an unstable gas that decomposes slowly in the gaseous phase to ordinary oxygen; the decomposition is slow at ordinary temperatures and low ozone concentrations but is greatly accelerated by heat. The homogeneous thermal gas phase decomposition of ozone can be described by a very simple mechanism:

$$M + O_3 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} O_2 + O + M \qquad -24.6 \text{ Kcal} \qquad 1$$

$$O + O_3 \xrightarrow[k_3]{} 2O_2 \qquad +93 \text{ Kcal} \qquad 2$$

where M represents all the substances (including ozone) present in the gas phase. Such substances could be O_2, N_2, CO_2, He or other "foreign" gases present in the ozone.[6] Each of the above gases has a different kinetic effect on the activation and deactivation of ozone. For dilute solution of ozone in oxygen, the rate of dissociation of ozone can be written:

$$-d(O_3)/dt = 2k_1k_3(O_3)^2/k_2(O_2)$$

thus, the more dilute the ozone, the slower the dissociation.[6] Axworthy and Benson[7] found that a sample containing 5% ozone in an atmosphere of oxygen could be stored at room temperature for nearly two months before the ozone concentration would fall below 4%.

However, both the mechanism and kinetics of the dissociation of ozone in water are uncertain. Weiss[3] observes that in increasingly alkaline solutions of ozone in water, absorption in the ultraviolet decreases, finally disappearing in strongly alkaline medium, at -40°C. On the basis of this observation, and the fact that decomposition increases rapidly with increasing alkalinity, Weiss proposed as a first step the reaction:

$$O_3 + OH^- \longrightarrow O_2 + HO_2 \qquad 3$$

being followed by the chain reactions:

$$O_3 + HO_2 \longrightarrow 2O_2 + OH \qquad 4$$

$$O_3 + OH \longrightarrow O_2 + HO_2 \qquad 5$$

$$HO_2 + HO_2 \longrightarrow O_3 + H_2O \qquad 6$$

$$HO_2 + OH \longrightarrow O_2 + H_2O \qquad 7$$

Weiss therefore calculated that the kinetics of ozone decomposition should be a 3/2 rate constant with respect to the ozone concentration. However, Alder and Hill[9] on the basis of their kinetic studies suggested a first order reaction with respect to ozone concentration and proposed the following mechanism as consistent with their results:

$$O_3 + H_2O \longrightarrow HO_3^{+} + OH^{-} \qquad 8$$

$$HO_3^{+} + OH^{-} \rightleftharpoons 2HO_2 \qquad 9$$

$$O_3 + HO_2 \longrightarrow HO + 2O_2 \qquad 10$$

$$HO_2 + HO \longrightarrow H_2O + O_2 \qquad 11$$

Recently Gorbenko-Germanov and Kozlova[10] have investigated the decomposition of ozone in basic aqueous media (-50^{0}C and 8M KOH) using the electron spin resonance technique and also absorption spectroscopy. By comparison with spectra obtained from potassium ozonide (KO_3) and potassium superoxide (KO_2), they were able to confirm the presence of the ozonide and superoxide radical -ion in 8M caustic potash solutions ozonized at 50^{0}C.[10, 11] On the basis of their studies, they suggested the following mechanism for ozone decomposition:

Step I (-50°C) $3O_3 + 2OH^- \longrightarrow 2O_3^- + H_2O + 2O_2$ 12

Step II (-50°C) $3O_3^- + H_2O \longrightarrow O_2^- + 3O_3 + 2OH^-$ 13

Step III (27.5°C) $2O_2^- + H_2O \longrightarrow HO_2^- + OH^- + O_2$ 14

Step IV (27.5°C) $HO_2^- \longrightarrow OH^- + \frac{1}{2}O_2$ 15

the overall reaction being $2O_3 \longrightarrow 3O_2$

Gorbenko-Garmanov and Kozlova[10] also suggested that the Step I was probably initiated by the following reaction:

$$O_3 + OH^- \longrightarrow O_3^- + OH \qquad 16$$

the hydroxyl radical then reacting further. This latter stage would explain the increased dissociation of ozone with increasing alkalinity.

Other studies have been carried out on the kinetics of ozone decomposition in water and Table 28 shows the range of variables covered and the varying conclusions concerning the reaction order relative to ozone.

Except for the work of Gorbenko-Germanov et al,[10,11] no direct studies (in comparison to kinetic study inferences) have been made of the decomposition of ozone in water. However, the reaction between water and ozone in the vapor phase has been investigated by Norrish et al,[21,22] who observed strong OH absorption bands in the flash photolysis of ozone-water mixtures and proposed that OH radicals are formed by reaction with the O radical:

$O_3 + h\nu \Longrightarrow O_2 + O$ 17

$O + H_2O \Longrightarrow 2OH$ 18

Based on reactions 1 and 18 it appears reasonable therefore to assume that an alternative mechanism for ozone decomposition in water might be:

$O_3 + H_2O \Longrightarrow O_2 + 2OH$ 19

Both Norrish and Wayne[22] and also Demore[23] have suggested that the hydroxy radical (OH) would then react as follows:

$OH + O_3 \Longrightarrow HO_2 + O_2$ 5

to produce the hydroperoxyl radical (HO_2) which could react:

$HO_2 + O_3 \Longrightarrow OH + 2O_2$ 4

similar to the reaction scheme previously proposed by Weiss.[8]

The reactions of the hydroxyl and hydroperoxyl radicals have been studied by investigators interested in the radiation chemistry of water. The hydroxyl radicals are reported to dimerize to form hydrogen peroxide:[24]

$OH + OH \Longrightarrow H_2O_2$ 20

and to further react with hydrogen peroxide:[24]

$OH + H_2O_2 \Longrightarrow HO_2 + H_2O$ 21

The hydroxyl and hydroperoxyl radicals are also reported to react with each other in the following manner:[25]

$OH + HO_2 \Longrightarrow H_2O + O_2$ 22

In alkaline solutions the following reaction becomes increasingly important:[26]

$$OH + OH^- \longrightarrow O^- + H_2O \qquad 23$$

The oxide radical, in contrast to the hydroxyl radical, can react with oxygen to form the ozonide ion:[27]

$$O^- + O_2 \longrightarrow O_3^- \qquad 24$$

As stated previously, the ozonide ion has been identified as an intermediate decomposition product of ozone in alkaline aqueous media (8M KOH) at below zero temperatures by investigation using the electron spin resonance technique.[10] These authors[11] also noted the presence of the superoxide radical ion (O_2^-) which had been previously suggested as a possible decomposition produce of the ozonide ion.[28] The half-life of the O_3^- radical at 25°C amounts to several milliseconds and is longer with higher pH[29] and concentration of oxygen in solution.[30] The mechanism of dissociation of the ozonide ion is uncertain, although it has been suggested that it dissociates to give oxygen and a peroxide ion[30] or by thermal dissociation back to the oxide ion (rate constant $3.3 \times 10^3 sec^{-1}$),[31] similar to that found by Garbenko-Germanov and Kozlova.[10] Like the hydroxyl radical, the hydroperoxyl radicals have also been reported to dimerize in aqueous solutions[25] as follows:

$$HO_2 + HO_2 \longrightarrow H_2O_2 + O_2 \qquad 25$$

disagreeing with reaction 6 as suggested by Weiss.[3] Taking into consideration the above mentioned reactions, the following stages for ozone decomposition

Table 28. SUMMARY OF THE KINETICS OF OZONE DECOMPOSITION IN WATER

Reference	pH range	Temp. range (°C)	Reaction order with respect to O_3
Rothmund et al[12]	2 → 4	0	2
Sennewald[13]	6.3 → 8	0	2
Weiss[8]	2 → 8	0	3/2
Adler & Hill[9]	1 → 2.8	0 → 27	1
Stumm[14]	7.6 → 10.4	1.2 → 19.8	1
Kilpatrick et al[15]	0 → 6.8	25	3/2
Kilpatrick et al[15]	13	25	2
Rankas et al[16]	5.4 → 8.5	5 → 25	3/2
Hewes & Davison[17]	2 → 4	30 → 60	2
Hewes & Davison[17]	6	10 → 50	3/2 → 2
Hewes & Davison[17]	6	10 → 20	1
Czapski[18]	10 → 13	25	1
Rogozhkin[19]	9.6 → 11.9	25	1
Merkulova et al[20]	0.22 → 1.9	5 → 40	1 or 2

in aqueous solution can therefore be suggested:

$$O_3 + H_2O \longrightarrow O_2 + 2OH \tag{19}$$

$$O_3 + OH_2 \longrightarrow O_2 + HO_2 \tag{5}$$

$$O_3 + HO_2 \longrightarrow O_2 + OH \tag{4}$$

$$OH + OH \longrightarrow H_2O_2 \tag{20}$$

$$OH + HO_2 \longrightarrow H_2O + O_2 \tag{22}$$

$$HO_2 + HO_2 \longrightarrow H_2O_2 + O_2 \tag{23}$$

$$OH + OH^- \longrightarrow O^- + H_2O \tag{24}$$

$$O^- + O_2 \longrightarrow O_3^- \tag{25}$$

the further dissociation of the ozonide ion probably following reaction 13 to 15. At high pH values the initiation state could well be:

$$3O_3 + 2OH^- \longrightarrow 2O_3^- + H_2O + 2O_2 \tag{12}$$

as demonstrated by Garbenko-Germanov and Kazlova,[10] followed by reaction 13 to 15.

As can be observed, the decomposition behavior of ozone in water is complicated depending on the alkalinity of the solution and possibly also on the oxygen content. All of the intermediate species formed are very reactive and possess vary short half-lives. Ivanov et al[32] have recently also proposed reaction 19 as a chain initiation step. However, since only an abstract was available to the author, it is difficult to know on what bases the proposed initiation step was suggested.

DISINFECTION POTENTIALS OF OZONE AND ITS DISSOCIATION SPECIES IN WATER

From the above survey, the possible species to be found in aqueous ozone solution are O_3, OH, HO_2, O^-, O_3^- and possibly the free oxygen atom if the ozone decomposes as in reaction 1 before reacting with the water. While Morris has stated,[33] and correctly, that there should be no relationship between the oxidation potential of a substance and its germicidal activity, however, it can be stated that substances that do not possess a high oxidation potential will not be germidically active. Conversely, chemical species that have high oxidation potential *may* possess germicidal potential.

Since almost no direct studies (except for H_2O_2), as yet, have been carried out on the germicidal activity of the above species, it is of interest to consider from a theoretical point of view the possible disinfection potential of the various species present in ozonated water. Hydrogen peroxide can be discarded at once as being the responsible species for the strong germicidal activity of ozonated solutions due to its slow disinfection effectiveness.[34]

Ozone has one of the highest oxidation potentials known (2.07 volts in acidic solutions and 1.24 volts in basic solutions). However, Baxendale[35] gives a value for the oxidation potential of the hydroxyl radical as 2.8 volts at $H^+ = 1.0$ M and 1.7 V for the hydroperoxyl radical, which suggests that the OH radical in water *might* be the species responsible for the strong germicidal activity of ozonated solution and not the free ozone itself.

Comparison of the reactions of the hydroxyl radical and ozone indicate a strong similarity. Ozone is well known to reduce the organic carbon content of wastewater effluents[36] and a similar effect has been noted using the OH radical.[37] In the latter study, the hydroxyl radical was produced by using Fenton's reagent which is the reaction of ferrous ions with excess of hydrogen peroxide:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH \qquad 21$$

Likewise, the ozonation of phenol in aqueous solution has been shown to produce as intermediate products both catechol and o-quinine[38] and similar oxidation products were observed in the reaction between the hydroxyl radical and phenol.[39] In the oxidation of various amino acids both the ozone[40] and OH radical[41] show a preference for sulfur containing amino acids. An intermediate product in the oxidation of cysteine by both ozone and the OH radical has been demonstrated to be cysteine.[40,41] One of the few simple carbon-hydrogen bond ozonation reactions which has been studied in water solution is that of malonic acid $(HOOC)CH_2(COOH)$.[42] The ozone attacked the methylene group (CH_2) converting it to an alcohol (hydroxymalonic acid) and a ketone(ketomalonic acid) function. The hydroxyl likewise is known to react with fully saturated organic compounds by simple hydrogen abstraction to form water and a carbon radical.[41] Thus, the reaction of the hydroxyl radical with malonic acid in aqueous solution could well proceed in a similar manner, i.e.,

$$(HOOC)\ CH_2(COOH) + OH \longrightarrow (HOOC)\ CH\ (COOH) + H_2O$$

$$(HOOC)\ CH\ (COOH) + OH \longrightarrow (HOOC)\ CH\ (OH)\ (COOH)$$

, hydroxy-malonic acid

Malonic acid is known to react readily with the OH radical,[43] however, no study was made of the resulting by-products.

Recently Hoigne et al,[44,45] have shown experimentally that as the pH increases the kinetics of ozonization of organic materials changes and that the relative reaction rates for the competing oxidation reaction in alkaline solutions is similar to that obtained in radiation studies, thus implying that the same species present in irradiated water is present in ozonated alkaline aqueous solution--namely the hydroxide ion, probably in the manner suggested by Garbenko-Germanov et al (reaction 16).

The chemical reactivity of the basic form of the hydroxyl radical, the oxide radical ion O^-, differs markedly from that of the OH in many reactions. While the hydroxyl radical adds readily to aromatic molecules, the reactivity of O^- toward aromatic compounds is lower by at least three orders of magnitude in the specific rate constant.[30] The OH radical is much more effective than is the oxide radical ion in oxidizing a number of inorganic anions.[41] Hydrogen abstraction reactions of O^-, on the other hand, exhibit a specific reactivity only slightly lower than that of OH.[30]

The limited studies on the ozonide radical[30] have shown that it is almost totally unreactive to aromatic molecules (such as the benzoate ion) as well as to methanol and ethanol. Likewise, the hydroperoxyl radical (HO_2)

has been shown to be almost inert in aqueous solution towards organic substances such as ascorbic acid[46] or cysteine.[47]

There are a number of facts that seem to indicate that the dissociation product(s) are more potent oxidants than the ozone itself. Hewes and Davison[36] have shown that the speed of ozonation of organic compounds in wastewater is pH dependent and increases with increasing temperature. Further, the oxidation process can be catalysed by adding certain inorganic cations. The above factors all affect the ozone decomposition rate and thus it appears that it is the decomposition products that affect the oxidation rate. Likewise, Reicherter and Sontheimer[48] have shown that both pH and catalysts affect the rate of ozone purification of wastewater systems and that a radical mechanism is probably responsible for ozone oxidation in aqueous solutions.

A limited number of preliminary experiments have been carried out in our laboratories to investigate the effect of pH on ozone inactivation of poliovirus I in aqueous solutions. For those experiments in the acidic pH range the method used was identical to those described in this report (see Section XIII). the virus being added to an ozonated solution already of the required pH. However, for those measurements carried out at pH greater than 8 a variation in the above method was necessary due to the rapid decomposition of the ozone. A 400 ml sample of unbuffered water was prepared containing the required amount of ozone. Then one millilitre of the virus suspension was added simultaneously with a sufficient quantity of concentrated sodium hydroxide solution. The alkaline solution produced the required pH.

The results obtained at the various pH studied (2, 4, 8.5, 9.5, 10) showed the same two stage structure as previously reported at pH 7.[4] The first stage was short $\leq$10 sec, with a rapid virus kill, followed by a slow second stage. Increasing the ozone concentration between 0.08 mg/l to 0.2 mg/l had little effect on the inactivation rate. A graph showing inactivation of the virus versus pH after 10 sec and 3 min is shown in Fig. 11. The results show a definite dependence on pH with the slowest activation rate occuring at a pH around 4. A similar result was obtained by Reicherter and Sontheimer[48] for the ozonization of wastewater effluents at various pH values.

The above results would appear to agree with the conclusions of Reicherter and Sontheimer[48] and suggest that the pH change affects the ozone dissociation and that the species thus produced inactivate the virus. However, an alternative explanation may be possible. It is known that under various conditions clumping of viruses occurs, and it is possible that clumps are broken up in the higher pH solution, and thus making them more susceptible to ozone inactivation.

In order to evaluate the above point, an inorganic catalyst was added at pH 4 to see if this affected the inactivation rate. It was observed

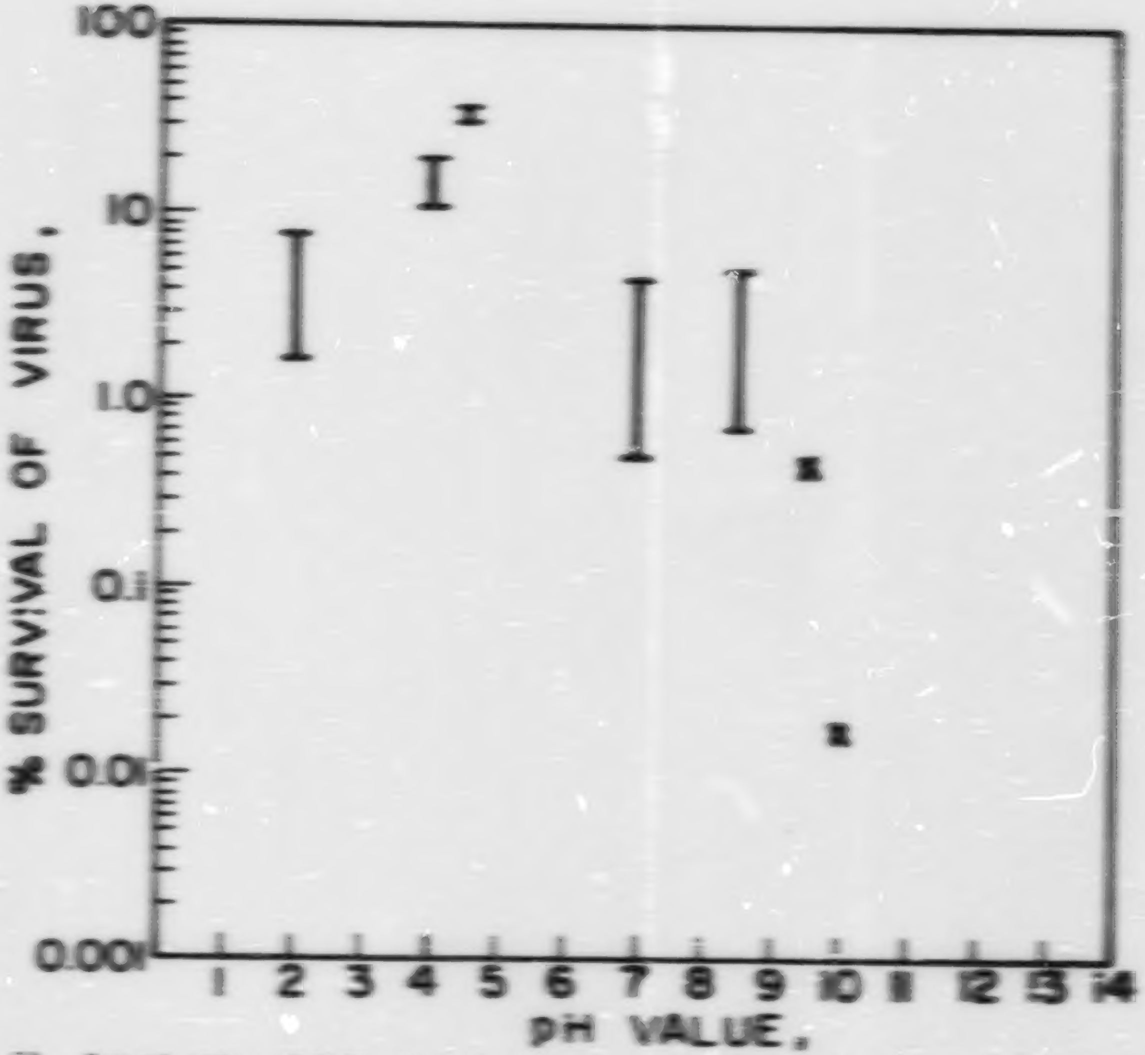


Fig. 11. Inactivation of Poliovirus 1 by ozone after 10 sec of various pH values (T=5°C)

that the addition of 10 mg Fe_2O_3/100 ml solution greatly increased the ozone decomposition rate and also the virus inactivation rate. The survival rate for the virus was decreased by a factor of about ten. Control experiments showed that Fe_2O_3 alone did not affect the virus stability.

In conclusion it therefore appears that it is the decomposition products of ozone that affect primarily the inactivation rate of viruses. However, while the ozone concentration decreases as quickly on addition of Fe_2O_3 as at pH=10, a smaller inactivation rate was observed. Thus it may be that at different pH values different species are formed and these species have different inactivation abilities.

DISCUSSION AND CONCLUSIONS

In conclusion, it appears as if the dissociation products of ozone in water may be more powerful oxidization agents than ozone itself. Due to the similarity between the reactions of organic compounds with ozone in aqueous solution and with the hydroxyl radical, it appears that it may be the hydroxyl radical that is mainly responsible for the high oxidative potential of ozone in water. Likewise the hydroxyl radical may be thought of as giving rise to the high germicidal action of ozone. However, this last statement must be treated with caution. Hoigne[49] has shown that the hydroxyl radical will react primarily with an organic solute when this is in solution together with a microorganism. Thus while in clean water systems the hydroxyl radical may well be available to inactivate viruses, once organic substances are present they will preferentially react with any high reactive species (such as OH) produced by the ozone on decomposition in water.

In summary, more experimental work, such as is presently being carried out in our laboratories is obviously needed before more definitive statements can be made about the germicidal properties of ozone and its dissociation species in water. An attempt must be made to further examine the effect of pH changes and increased catalytic decomposition of the ozone on the disinfection ability of ozone. Further studies need to be initiated, if possible, on the direct effect of the dissociation species of ozone on disinfection, such as OH, HO_2, O_3^-, O_2^- and O^-.

REFERENCES

1. Guinverch, P. Three Years of Ozone Sterilization of Water in Paris. In: Ozone Chemistry and Technology, Advances in Chemistry Series No. 21, 1959. p. 416-429.

2. Lebout, H. Fifty Years of Ozonation at Nice. In: Ozone Chemistry and Technology, Advances in Chemistry Series No. 21, 1959. p. 450-452.

3. Draper, E.W.J. Practical Aspects of Water and Waste Water Treatment by Ozone. In: Ozone Water and Wastewater Treatment, Evans III, F.L. (ed.). Ann Arbor Science, 1972. p. 146-179.

4. Katzenelson, E., B. Kletter, H. Schecter and H.I. Shuval. Inactivation of Viruses and Bacteria by Ozone. In: Chemistry of Water Supply, Treatment and Distribution, Rubin, A.J. (ed.). Ann Arbor Science, 1974. p. 409-421.

5. Kinman, R.N., A.P. Black, and W.G. Thomas, Jr. Studies of Water Disinfection with Iodine. National Specialty Conference on Disinfection, Amer. Soc. of Civil Engr., p. 11-35, 1970.

6. Benson, S.W. and A.E. Axworthy, Jr. Implications of Data on the Gas Phase Decomposition of Ozone. Advances in Chemistry Series No. 21, 1959. p. 398-404.

7. Axworthy, A.E., Jr. and S.W. Benson. Mechanism of Gas Phase Decomposition of Ozone. In: Thermal and Photochemical Reactions Advances in Chemistry Series No. 21, 1959. p. 388-397.

8. Weiss, J. Investigations on the Radical HO_2 in Solution. Trans. Far. Soc. 31:668-681, 1935.

9. Adler, M.G. and G.R. Hill. The Kinetics and Mechanism of Hydroxide Ion Catalyzed Ozone Decomposition in Aqueous Solution. J. Amer. Chem. Soc. 72:1884-1886, 1950.

10. Gorbenko-Germanov, D.S. and I.V. Kozlova. Mechanism of Ozone Decomposition in Alkaline Aqueous Media. Dokl. Akad. Nauk. USSR, 210:851-854, 1973.

11. Gorbenko-Germanov, D.S. and I.V. Kozlova. Intermediate Decomposition Products of Ozone in Alkaline Aqueous Media Investigated by Electron Spin Resonance. Zhur. Fiz. Khim. 48:166-168, 1974.

12. Rothmund, V. and A. Burgstaller. Decomposition Velocity of Ozone in Aqueous Solutions. Monatsch Chem. 34:665-692, 1913.

13. Sennewald, K. Decomposition of Ozone in Aqueous Solutions. Z. Phys. Chem. (Leipzig) A164:305-317, 1933.

14. Stumm, W. The Solubility of Ozone in Aqueous Solution. Helvetica Chimica Acta, 37:773-778, 1954.

15. Kilpatrick, M.L.C, C.C. Herrick, and M. Kilpatrick. The Decomposition of Ozone in Aqueous Solutions. J. Amer. Chem. Soc., 78:1784-1789, 1956.

16. Rankas, M.M., E.K. Siirode, and S.R. Kyulm. Rate of Ozone Decomposition in Different Waters. Trudy Tallinskoga Politeknich Inst. Ser. A, 198:219-232. Chemical Abstracts 60:2633e

17. Hewes, C.G. and R.R. Davison. Kinetics of Ozone Decomposition and Reaction with Organics in Water. AIChE Journal 17:141-147, 1971.

18. Czapski, G., A. Samuni, and R. Yellin. The Disappearance of Ozone in Alkaline Solution. Isr. J. Chem. 6:969-971, 1968.

19. Rogozhkin, G.I. Kinetics of Ozone Absorption by Alkaline Solutions. Chem. Abs. 73:91108y.

20. Merkulova, V.P., V.S. Lovchikov, and M.D. Ivanovskii. Kinetics of the Breakdown of Ozone in Sulfate Solutions. Izv. Vyssh. Ucheb. Zaved. Khim. Tekhnol. 14:(6) 818-823, 1971.

21. McGrath, W.D. and R.G.W. Norrish. Influence of Water on the Photolytic Decomposition of Ozone. Nature 182:235-237, 1958.

22. Norrish, R.G.W. and R.P. Wayne. The Photolysis of Ozone by Ultraviolet Radiation II. The Photolysis of Ozone Mixed with Certain Hydrogen-Containing Substances. Proc. Roy. Soc. Ser. A. 288:361-373, 1965.

23. Demore, W.B. Rate Constants for the Reactions of Hydroxyl and Hydroperoxyl Radicals with Ozone. Science, 180:735-737, 1973.

24. Thomas, J.K. Rates of Reaction of the Hydroxyl Radical. Trans. Far. Soc., 61:702-707, 1965.

25. Czapski, G. and B.H.J. Bielski. The Formation and Decay of H_2O_3 and HO_2 in Electro-Irradiated Aqueous Solutions. J. Phys. Chem. 67:2180-2184, 1963.

26. Hochanadel, C.J. Photolysis of Dilute Hydrogen Peroxide Solutions in the Presence of Dissolved Hydrogen and Oxygen. Radiat. Res. 17:286-301, 1962.

27. Adams, G.E., J.W. Boag and B.D. Michael. Rate Constant for Reaction of O^- with Oxygen. Nature, 205:898-899, 1965.

28. Felix, W.D., B.L. Gall and L.M. Dorfman. Pulse Radiolysis Studies. IX.Reactions of the Ozonide Ion in Aqueous Solutions. J. Phys. Chem. 71: 384-392, 1967.

29. Czapski, G. and L.M. Dorfman. Pulse Radiolysis Studies. V. Transient Spectra and Rate Constants in Oxygenated Aqueous Solutions. J. Phys. Chem. 68:1169-1177, 1964.

30. Czapski, G. Pulse Radiolysis Studies in Oxygenated Alkaline Solutions. J. Phys. Chem. 71:1683-1688, 1967.

31. Gall, B.L. and L.M. Dorfman. Pulse Radiolysis Studies, XV. Reactivity of the Oxide Radical Ion and of the Ozonide Ion in Aqueous Solution. J. Amer. Chem. Soc. 91:2199-2204, 1969.

32. Ivanov, Yu. E., G.P. Nikitina, M.F. Pushlenkov and V.G. Shunkov. Ozone in Aqueous Solutions. I.Kinetics and Mechanism of the Decomposition of Ozone in Water and in Nitric Acid Solutions. Zhur.Fiz. Khim. 46:2163, 1972.

33. Morris, J.C. Disinfectant Chemistry and Biocidial Activities. In: National Specialty Conference on Disinfection. Amer. Soc. of Civil Eng., 1970. p. 609-633.

34. Yoshpe-Purer, Y. and E. Eylan. Disinfection of Water by Hydrogen Peroxide. Health Laboratory Science 5:233-238, 1968.

35. Baxendale, J.H. Effects of Oxygen and pH in the Radiation Chemistry of Aqueous Solutions. Radiat. Res. Suppl. 4:114-135, 1964.

36. Hewes, C.G. and R.R. Davison. Renovation of Wastewater by Ozonation. AIChE Symposium Series 69:71-80, 1972.

37. Bishop, D.F., G. Stern, M. Fleischmann, and L.S. Marshall. Hydrogen Peroxide Catalytic Oxidation of Refractory Organics in Municipal Waste Waters. I&EC Proc. Des. & Develop. 7:110-113, 1968.

38. Eisenhauer, H.R. The Ozonization of Phenolic Wastes. J. WPCF. 40:1887-1899, 1968.

39. Stein, G. and J. Weiss. Some Free Radical Reactions of Phenol. The Action of Hydrogen Peroxide-Ferrous Salt Reagent and of X-Rays on Aqueous Solutions of Phenol. J. Chem. Soc. 3265-3274, 1951.

40. Mudd, J.B., R. Leavitt, A. Ongun, and T.T. McManus. Reaction of Ozone with Amino Acids and Proteins. Atmos. Envir. 3:669-681, 1969.

41. Dorfman, L.M. and G.E. Adams. Reactivity of the Hydroxyl Radical in Aqueous Solutions. U.S. Department of Commerce, National Bureau of Standards, NSRDS-NBS46, 1973

42. Dobinson, F. Ozonation of Malonic Acid in Aqueous Solution. Chemistry and Industry, 1959, p. 853-854.

46. Barr, N.F. and C.G. King. The X-Ray Induced Oxidation of Ascorbic Acid and Ferrous Ion. J. Amer. Chem. Soc. 78:303-305, 1956.

47. Swallow, A.J. The Action of X-Radiation on Aqueous Solutions of Cysteine. J. Soc. 76:1334-1339, 1952.

48. Reicherter, A. and H. Sontheimer. Investigation of the Use of Ozone for Water and Wastewater Purification. Vom Wasser. 41:369-380, 1973.

49. Hoigne, J. Aqueous Radiation Chemistry in Relation to Waste Treatment. IAEA Symposium, Munich. SM-194-401, March, 1975.

43. Scholes, G. and R.L. Wilson. Radiolysis of Aqueous Thymine Solutions. Determination of Relative Reaction Rates of OH Radicals. Trans. Far. Soc. 63:2983-2993, 1967.

44. Hoigne, J. and H. Bader. The Rate of the Hydroxyl Radical in the Ozonization of Aqueous Solutions. Chimica. 29:20, 1975.

45. Hoigne, J. Ozone vs Irradiation Treatment of Organic Impurities in Water. IAEA Symposium, Munich. SM-194-410, March, 1975.

SECTION XI

SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF OZONE IN AQUEOUS SOLUTIONS

INTRODUCTION

The main reasons for developing methods of ozone determination were to study the natural occurence of ozone in the admosphere and its presence as a result of air pollution. The methods for the determination of atmospheric ozone involve a variety of analytical techniques. Chemical,[1-3] electrochemical,[4,5] and optical methods[6-10] have been developed, each of them with its advantages and disadvantages.

Although the use of ozone as a disinfectant for water was reported on as early as 1895,[11] its application in water purification has been more or less limited to Europe.[12] At present, ozone is actively being investigated for use in water and waste water treatment. Although this technique is very effective, so far no rational and scientific basis has been given for its practical application.

The impetus to develop a suitable procedure for the determination of dissolved ozone in water arose from the requirement of the kinetic study carried out in this laboratory for the inactivation of viruses and bacteria by ozone. For this purpose, the method must be able to detect ozone concentrations as low as 0.01 ppm and should also use small samples in order to overcome the problem of overly large volumes in the inactivation reactor vessel. The method should be sufficiently rapid so as to allow for frequent sampling in the course of kinetic experiments.

Since many methods for ozone determination in air have been previously employed, a survey of the pertinent literature has been made and the iodide chemical method selected. The iodometric method is accepted as the method of reference, since it agrees with the absolute physical methods based on gas density[13] or pressure change measurements.[14]

The classical procedure described in Standard Methods[15] for the determination of residual ozone in water, requires large volumes of sample under normal conditions and would require samples as large as 1 liter for the detection of ozone concentration as low as 0.03 ppm. Moreover, thiosulfate titration of iodine liberated by ozone has also some limitations.

To avoid these difficulties, the spectrophotometric method initially reported upon by Salzman[16] for the determination of ozone in small volumes of water was evaluated and refined. This involves oxidation of a buffered iodide solution and spectrophotometric measurement of the triiodide ion liberated by ozone. The concentration of iodide and the pH of the solution have a marked effect on the results.

The recommended method corresponds to the requirements mentioned earlier for our experimental conditions. In addition, it has the advantage of allowing the preparation of a calibration graph with a stable iodine solution, instead of using the unstable and reactive solution of ozone in water, as a standard.

MATERIALS

Apparatus

Ozonizer -

The ozone is generated from air in a Fischer-Laboratory Ozonator (OZ III) based on the electrical discharge method. An appropriate suction pump is used for drawing the air through the ozonator. The air stream is dried initially by means of a "Koy Senior" air purifier and flow equalizer, and by means of two columns containing granulated potassium hydroxide and silica gel, respectively. The ozone generated by this equipment is passed through a sintered glass dispersion tube (Corning grade) into water, contained in a two liter Pyrex glass reactor. The tubing used is of Teflon or glass, since these materials have the least effect on ozone.

Spectrophotometer -

Zeiss P. MQII, equipped with stoppered 20 and 40 mm quartz cuvettes, suitable for use in the U.V. region.

Glassware -

It is very important to keep the glassware scrupulously clean, since traces of impurities may cause very serious errors. In the preparation of ozone-demand free glassware, all the glassware used in the tests is cleaned with dichromate-concentrate sulfuric acid mixture, rinsed in tap water, then by distilled water, soaked in a strong ozone solution and finally dried at 180^{0}C to ensure the elimination of ozone. Only experiments conducted in a clean, ozone demand free system, would expose viruses to a relatively constant concentration of ozone during the course of the experiment.

Reagents

Chemicals -

Highest quality analytical grade chemicals are used.

Distilled Water -

Distilled water of high purity must be used; demineralized water from a mixed-bed ion exchanger is not suitable for this work. The distillation must be carried out in an all glass still, in the presence of alkaline potassium permanganate and redistillation is necessary. The dilution water used has to be ozone-demand free. For this, it is convenient to ozonize bidistilled water and then to ensure its dissipation by boiling.

Standard iodine solution 0.01 N -

Dissolve successively 6.4 g potassium iodide and 1.2692 g iodine, to a volume of 1000 ml. It is advisable to mix the solid iodine and potassium iodide in a relatively small volume of water until the iodine is completely dissolved, then to dilute the solution to 1000 ml. Age one day before use. The solution may be standardized by titration with arsenious oxide. One ml of 0.01 N iodine (triiodide) is equivalent to 24 μg ozone. A standard iodine solution 0.01 N may be alternatively prepared from an ampoule.

Neutral potassium iodide reagent -

Dissolve 13.61 g potassium dihydrogenphosphate, 14.20 g anhydrous disodium hydrogen phosphate, and 20.0 g potassium iodide. Bring mixture to 1000 ml. This solution must be stored in a dark bottle in the refrigerator and must avoid exposure to sunlight. This reagent is used in the procedure "B" for high ozone concentrations. In the procedure "A" for low ozone concentrations, the same reagent is used, but with a higher concentration of potassium iodide: 5%, instead of 2%.

METHOD

Ozone reacts with the neutral potassium iodide solution and liberates iodine and in an excess of potassium iodide, iodine is in the complexed triiodide form. The concentration of triiodide liberated is determined spectrophotometrically at a wave length of 352 mμ.

Procedure

Two similar procedures are recommended: procedure "A" for low ozone concentrations (0.01 to 0.30 ppm) and procedure "B" for high concentrations (0.30 to 2.0 ppm).

In procedure "A", 10 ml of the ozone containing samples are introduced into a test tube which contains 2 ml of 5% neutral potassium iodide

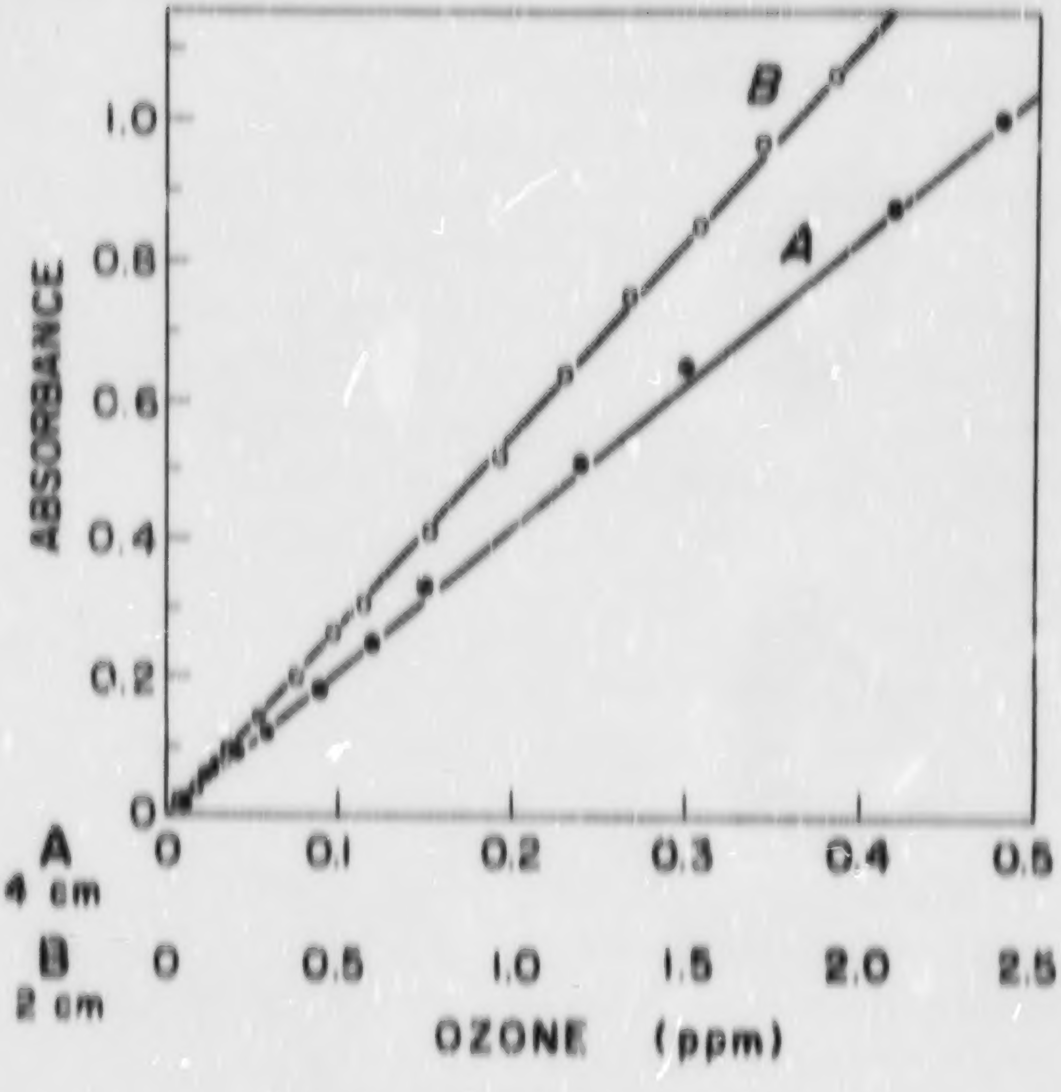


Fig. 12. Calibration curve for determination of Ozone in water

buffered reagent. The test tube is left for about 30 minutes in a cool and dark place. Triiodide is quantitatively liberated and the intensity of absorbance is then read, using cells of 40 mm light path, against a distilled water blank.

In procedure "B" for higher ozone levels, 5 ml of the sample are introduced in a test tube containing 5 ml of 2% neutral potassium iodide. After about 30 minutes, the intensity of the absorption is read using cells of 20 mm light path.

Calculation

The stoichiometry of the ozone iodide reaction, upon which all calculated ozone concentrations are based, is 1:1, one mole of ozone liberates one mole of iodine at neutral pH.[1,17,18]

Calibration graph

A standard curve of absorbance versus iodine (or ozone) concentration is plotted from readings of a series of freshly prepared standards. The stock iodine solution 0.01 N (1 ml = 240 μg of ozone) is diluted with neutral potassium iodide reagent to obtain a solution of iodine 0.00004 N (1 ml = 0.96 μg ozone). Different portions of this solution are transferred to test tubes and diluted with neutral potassium iodide reagent to 12 ml final volume (curve A) or to 10 ml final volume (curve B). Mix and immediately read the absorbance at 352 mμ, using distilled water as the reference. Calibration graphs for both procedures are shown in Fig. 12. Beer's Law is obeyed over the whole range investigated in both cases.

RESULTS AND DISCUSSION

Procedural Variables

An evaluation of several procedural variables was undertaken in order to ascertain the effects they would have on ozone determination.

Concentration of Potassium Iodide Reagent

The effect of different concentrations of potassium iodide on the intensity of the colour development was investigated. Table 29 presents the absorbance values at different ozone levels utilizing solutions of 2%, 5% and 10% potassium iodide concentration. The obtained values are comparable, but there are increasing absorbance values with higher potassium iodide concentrations, especially at the low ozone concentration levels.

Changing the concentration of the phosphate buffer affects the stoichiometry of the ozone-iodide reaction, Scott[19] found lower ozone values by using a weaker buffered solution, possibly by allowing a rise of the pH during the reaction of ozone and potassium iodide.

Stability of the Colour Intensity

The rate of colour development was also checked at five minute intervals between time zero and one hour. The results are presented in Fig. 13. By using a 2% potassium iodide solution, the intensity of the colour increases slightly during the first 25-30 minutes and then begins to fade slowly. More than 90% of the iodine is liberated at time zero

Table 25. INFLUENCE OF POTASSIUM IODIDE CONCENTRATION ON INTENSITY OF COLOUR

Potassium Iodide concentration	Absorbance (Immediate lecture) a*	b*	c*
2%	0.060	0.205	0.505
5%	0.070	0.210	0.515
10%	0.075	0.220	0.525

* Different ozone concentrations levels

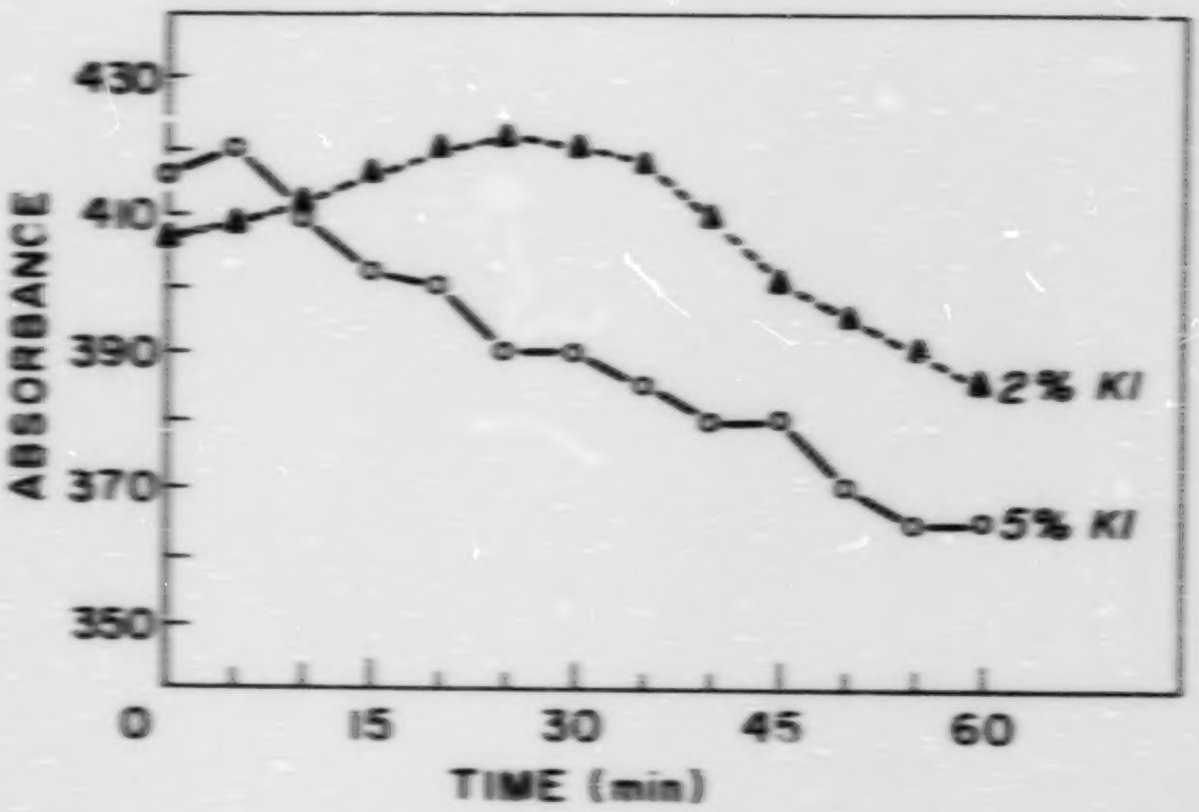


Fig. 13. Stability of colour intensity as function of potassium iodide concentration

and the remainder is liberated during the first half hour. By using a more concentrated potassium iodide solution (5%), almost all the iodine is liberated during the first five minutes, then the colour begins to fade gradually.

Since our investigations require multiple determinations to be performed during the first fifteen minutes of ozone action, optimal results are obtained by using the 2% potassium iodide solution and reading the absorbance approximately 30 minutes after the triiodide ion is liberated by ozone.

METHOD EVALUATION

Reproducibility

In order to establish the precision of the proposed method for determination of ozone in water, six parallel determinations were carried out, in a consecutive order, at different ozone concentrations. The standard deviation of the results and the 95% confidence limits are calculated and shown in Table 30. The ozone concentration levels examined are 0.05 to 0.33 ppm in the procedure used for low ozone levels and 0.23 to 1.92 ppm in that used for higher ozone levels. The fluctuations of the results are minimal (less than 4%) when the procedure used corresponds to the ozone level for which it is intended. For instance, at 0.20 ppm level, the relative standard deviation is 0.9% with the suitable procedure "A" and 3.4% if procedure "B" is used.

Table 30. REPRODUCIBILITY OF RESULTS IN THE SPECTROPHOTOMETRIC METHOD

Procedure	Mean Value[a] O_3 ppm	Range of Results min. and max.	Standard Deviation ppm	Standard Deviation %	95% Confidence limits
A	0.058	0.055-0.060	0.002	3.4	0.0020
	0.125	0.120-0.130	0.003	2.4	0.0027
	0.217	0.216-0.220	0.002	0.9	0.0012
	0.329	0.327-0.331	0.002	0.6	0.0015
B	0.236	0.23-0.25	0.008	3.4	0.0063
	0.418	0.41-0.43	0.008	1.7	0.0057
	1.077	1.06-1.09	0.008	0.8	0.0075
	1.920	1.90-1.95	0.024	1.2	0.0180

[a] Six replicates at each level

Sensitivity

The sensitivity of the spectrophotometirc method applicable to low ozone levels is between the limits 0.01 to 0.30 ppm. For the method of higher ozone levels, it is between 0.05 to 2.00 ppm.

Correlation with Other Methods for Ozone Determination

The results obtained from spectrophotometric ozone determination using the neutral potassium iodide reagent were compared with those obtained using the Mast reagent and also with the standard volumetric method.

The Mast reagent* recommended for the Mast Ozone Meter** was used by Jones, et al[20] in a toxicological experimental study. In this method, ozone reacts initially with the potassium bromide solution and the liberated bromine then reacts with potassium iodide; the free iodine is then measured spectrophotometrically.

Repeated determinations were carried out comparing the neutral potassium iodide reagent with the Mast reagent, at different ozone levels. No significant differences were observed.

The ozone determination in the standard volumetric[15] as well as in the spectrophotometric method[16] is based on the measurement of the triiodide ion liberated by ozone from potassium iodide solution. In the spectrophotometric method the amount of triiodide ion is measured directly. The precision and sensitivity of this instrumental analysis permits it to be chosen as an independent variable. On the other hand, in the volumetric method, after the liberation of triiodide ion by ozone, two additional variables are introduced. These are (1) acidification, in order to obtain the well defined stoichiometry of the triiodide-thiosulfate reaction, and (2) end point detection, by formation of starch iodine complex. To compare the results obtained by these two methods parallel samples of different ozone concentrations were analysed carefully by both procedures. The results are presented in Table 31.

---

* Mast Reagent: 10 gr potassium iodide; 25 gr potassium bromide; 1.25 g sodium phosphate monobasic and 5.0 g sodium phosphate dibasic; made up to 500 ml with distilled water.

** Mast Development Company, Davenport, Iowa.

The comparative results obtained by both methods show the volumetric readings to be greater than those of spectrophotometry. A reasonable explanation of this difference can be derived with reference to the stoichiometric reaction:

$$O_3 + 2KI + H_2O \rightleftharpoons I_2 + O_2 + 2KOH$$
$$I_2 + I^- \longrightarrow I_3^-$$

The liberated oxygen O_2, which appears here as a by product, does not modify the absorbance readings in the spectrophotometric method, at neutral pH. Since in the volumetric method acidification is used, the O_2 supplied by the mechanism of the reaction may liberate supplementary I_3^- ions:

Table 31. COMPARISON OF TITRIMETRIC AND SPECTROPHOTOMETRIC METHODS FOR DETERMINATION OF OZONE IN WATER

Ozone Level Range ppm	Number of samples	Mean of results, ppm	
		Standard method	Spectrophotometric method
0-0.49	5	0.33	0.28
0.5-0.99	8	0.80	0.74
1.0-1.99	12	1.44	1.37
2.0-4.99	15	3.97	3.76
Total	40		

$$6I^- + O_2 + 4H^+ \rightleftharpoons 2I_3^- + 2H_2O$$

which are subsequently measured and serve as a source of error.

It is known,[21, 22] that even the dissolved atmospheric oxygen may be a potential source of error in the volumetric titration of iodine, but fortunately this reaction has a low velocity. However, it may be catalyzed by light, metal ions or other factors. In the case of ozone determination, the newly formed oxygen may be in an activated form or some other intermediate species may also be produced, which in acid solution could liberate additional iodine.

If the reaction between ozone and iodide takes place via some intermediate forms, i.e. ozonide ion O^-_3, as some authors have reported,[23] the stoichiometry of the reaction in neutral medium remains one to one, in agreement with the classical equation:

$$O_3 + I^- \longrightarrow O_3^- + 1/2\ I_2$$

$$O_3^- + I^- + H_2O \longrightarrow 2OH + O_2 + 1/2\ I_2$$

However, if the same reaction takes place in acidic-medium, the ozonide ion may be transformed to HO_3^-, which may be able to oxidize additional iodide to iodine, i.e.,

$$H^+ + O_3^- \rightleftharpoons HO_3$$

$$HO_3 + I^- \longrightarrow HO_3^- + 1/2\ I_2$$

$$HO_3^- + I^- + 2H^+ \longrightarrow HO_2 + H_2O + 1/2\ I_2$$

Thus, in the above presented two mechanisms, the classical equation and the ozonide pathway, additional iodine is liberated in the standard volumetric determination. This fact correlates with our observations.

The second possible source of error in the titrimetric standard method is the end point detection. The starch end point is not sufficiently sensitive for titration with very dilute thiosulfate solution. Furthermore, the sensitivity of this end point detection depends also on the amount of iodide ion present in solution.

APPLICATIONS FOR INACTIVATION STUDIES

The inactivation process is an interaction between germicidal agent and microorganism, analogous to a chemical reaction, that is assumed to follow the course of a first order reaction. The classic Chick's law of germicidal action is valid when only the microorganism is the critical reactant. The practical approach to this condition would be to ensure that the concentration of the disinfectant and the other milieu factors remain constant during the period of exposure.

Different experiments were carried out in order to optimize these working parameters for inactivation experiments using ozone as a germicidal agent.

One of the difficulties in the work with ozone is its low stability in water solution. Alder and Hill[24] and Stumm[25] showed that ozone in solution is more stable at temperatures near the freezing point of water. To better understand the conditions required to obtain stable ozone concentrations the following variables were examined: the influence of temperature and the influence of stirring rate as a function of time and of ozone concentration.

The Influence of Temperature

Fig. 14 presents the influence of temperature on stability of ozone concentration versus time. The two levels were those generally used in the study of ozone's disinfecting activity. It appears that at 5°C the ozone solution is evidently more stable than at 22°C. The concentration of ozone solution at 5°C remains almost constant during the first five minutes and decreases slightly during the following 15-20 minutes. At room temperature (22°C) ozone decay begins immediately and becomes significant after the first five minutes.

The Influence of Stirring Rate

Fig. 15 presents the influence of stirring rate on ozone stability in kinetic experiments, at three ozone levels: 0.1, 0.6, and 1.0 ppm. Stirring is at 60, 80 and 100 rpm, while maintaining a constant temperature of 5°C in a buffered solution of pH 6.8.

The kinetics of ozone decomposition under these conditions was found to be correlated with the rate of stirring and the concentration of ozone. The percentage of loss in ozone rises with the increase in ozone

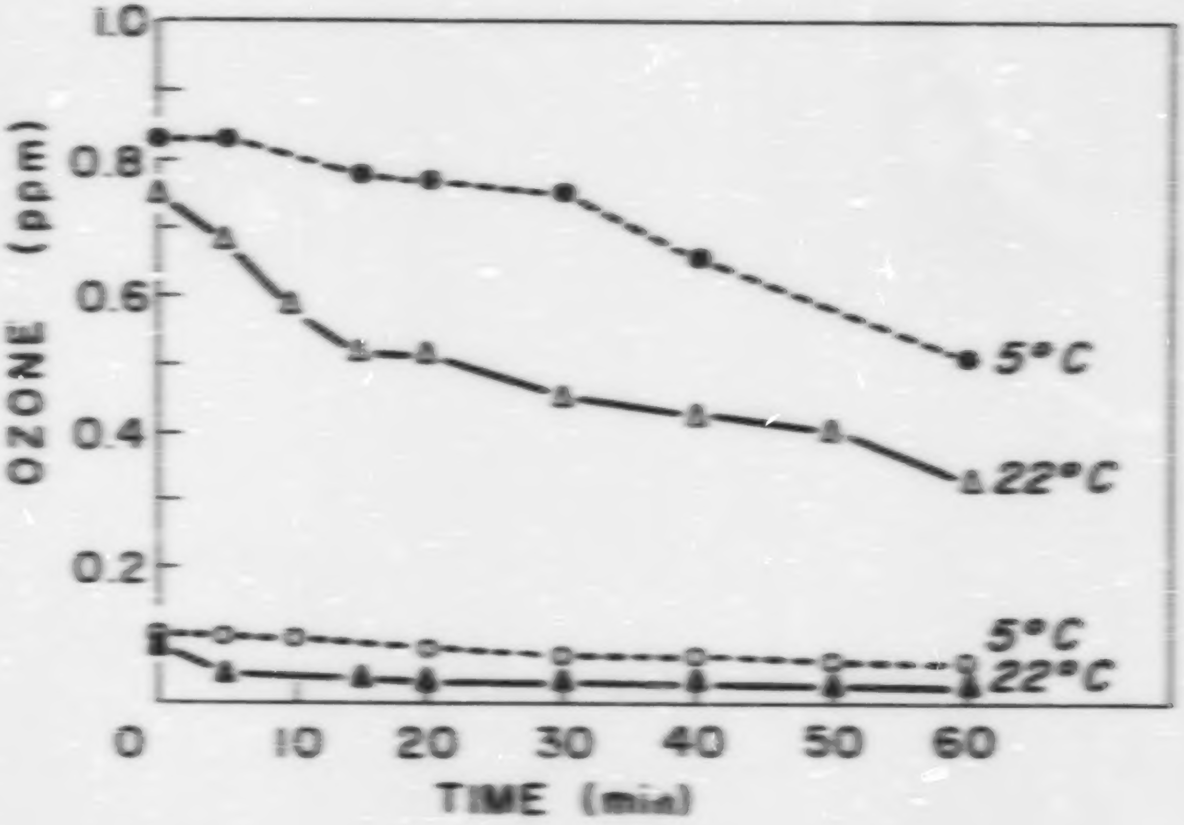


Fig. 14. Ozone aqueous solution stability versus time, temperature and concentration

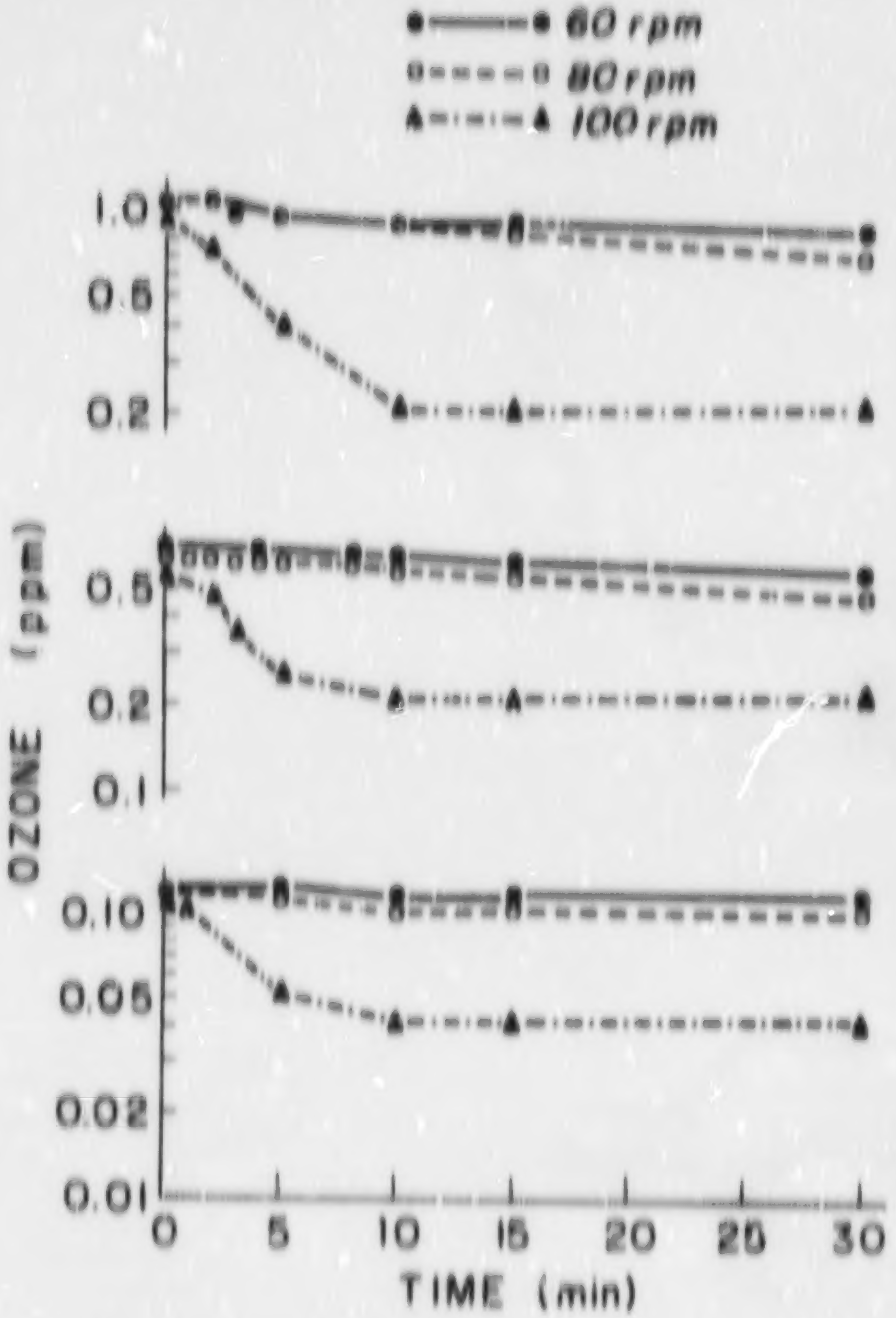


Fig. 15. Influence of stirring rate on Ozone stability (at 5°C)

concentration. Although the stirring at 60 and 80 rpm slightly reduces the stability of the ozone solution, the effect of 100 rpm is far more accentuated. During 15 minutes, at 60 and 80 rpm, the loss of ozone is loss of ozonu is less than 15%. At 100 rpm, the loss of ozone is 30-40% after only 2-3 minutes and after 15 minutes this has reached 75-80%.

It may be concluded that for an experiment of 15 minutes duration, 80 rpm is the optimal stirring rate for these conditions.

Ozone Demand of Virus Culture Suspensions

The stability of ozone concentration may also be influenced by the presence of organic matter in virus culture suspensions. A partially purified poliovirus culture was added, in different dilutions, to ozone solutions at 5^{0}C and at a stirring rate of 80 rpm. The residual ozone was determined during a period of 15 minutes. The results are presented in Fig. 16. After a ten minute contact of the partially purified virus with the ozone solution, the loss of ozone is about 90% for virus dilution of 5.10^{-3}, 75% for virus dilution of 5.10^{-4} and 10% for a dilution of 5.10^{-5}.

Highly purified stock cultures, containing about 10^{8} pfu per ml, showed very low ozone demand even at high virus concentration, with the stock diluted as little as 10^{-3}.

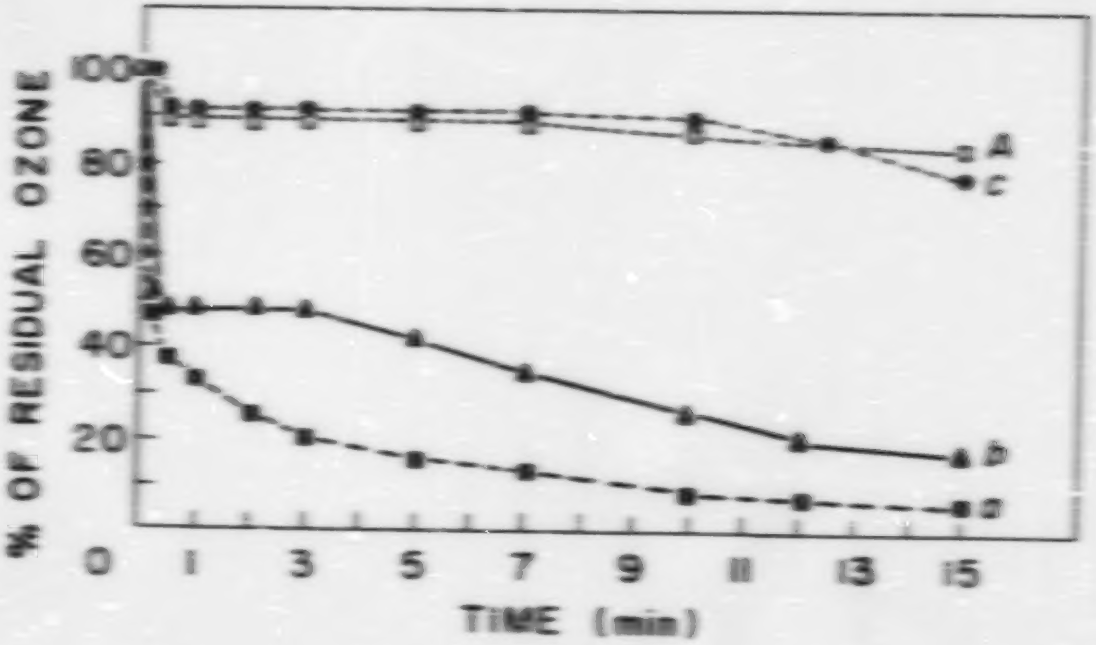


Fig. 16. Kinetics of ozone demand of different virus suspensions at 5°C and stirring rate 60 rpm.

a – Stock culture dilution 5×10^{-1};

b – Stock culture dilution 5×10^{-2};

c – Stock culture dilution 5×10^{-3};

A – Purified stock culture dilution 5×10^{-3}

As a result of these studies on the factors affecting the stability of the ozone solutions we were able to establish optimal conditions for our inactivation experiments.

REFERENCES

1. Byers, D.H. and B. Saltzman. Determination of Ozone in Air by Neutral and Alkaline Iodide Procedures. Advan. Chem. Ser. 21:93-101, 1959.

2. Hofmann, P. and P. Stern. Photometrische Bestimmung Niedriger Ozonkonzentrationen in Wassern. Anal. Chim. Acta 47:113-120, 1969.

3. Layton, R.F. and R.N. Kinman. A New Analytical Method for Ozone, Nationality Speciality Conference on Disinfection, Am. Soc. Civil Eng., p. 285, 1970.

4. Ehmert, A. Chemical Ozone Measurement. Advan. Chem. Ser. 21:128-132, 1959.

5. Hersh, P. and R. Deuringer. Galvanic Monitoring of Ozone in Air. Anal. Chem. 35:897-899, 1963.

6. Alway, C.D. and G. Slomp. Ozone Concentration Meters. Advan. Chem. Ser. 21:108-116, 1959.

7. Hanst, P.L., E. Stephens, W. Scott, and R. Doerr. Adsorption for the Infrared Determination of Trace Amounts of Ozone. Anal. Chem. 33:1113-1116, 1961.

8. Bersis, D. and E. Vassiliou. A chemiluminescence method for determining ozone. Analyst 91:499-505, 1966.

9. Amos, D. Specific Spectrofluorimetric Determination of Atmospheric Ozone Using 2-diphenylacetyl 1-3 Indandione 1 Hydrazone. Anal. Chem. 42:842-844, 1970.

10. Sachdev, S.L., J.P. Lodge, and P.W. West. Specific Method for the Determination of Ozone in the Atmosphere. Anal. Chim. Acta 58: 141-147, 1972.

11. Van Ermengen, E. Sterilization of Water by Ozone. Ann. Inst. Pasteur 9:673-680, 1895.

12. Hann, V.A. Disinfection of Drinking Water with Ozone. J.A.W.W.A. 46:1316-1320, 1956.

13. Boelter, E.D., C.L. Putnam and E. Lash. Iodometric Determination of Ozone of High Concentration. Anal. Chem. 22:1533-1535, 1950.

14. Birdsall, C.M., A.C. Jenkins, and E. Spadinger. Iodometric Determination of Ozone. Anal. Chem. 24:662-664, 1952.

15. Standard Methods for the Examination of Water and Waste Water, p. 271. 13th edn. APHA, AWWA, WPCF, New York, 1971.

16. Saltzman, B.E. and N. Gilbert. Iodometric Microdetermination of Organic Oxidants and Ozone. Anal. Chem 31:1914-1920, 1959.

17. Kopczynski, S.L. and J.J. Bufalini. Some Observations on Stoichiometry of Iodometric Analyses of Ozone at pH 7.0. Anal. Chem. 43:1126-1127, 1971

18. Hodgeson, J.A., R.E. Baumgardner, B.E. Martin and R.A. Rehme. Stoichiometry in the Neutral Iodometric Procedure for Ozone by Gas-Phase Titration with Nitric Oxide. Anal. Chem. 43:1123-1126, 1971.

19. Scott, D.W. Measurements of Total Oxidants in the Atmosphere. Report, Warren Spring Laboratory, England, 1970.

20. Jones, R.A., L.J. Jenkins, R.A. Coon, and J. Siegel. Effects of Long Term Continuous Inhalation of Ozone on Experimental Animals. Toxicol. Appl. Pharmacol. 17:189-202, 1970.

21. Skoog, D.A. and D.M. West, Fundamentals of Analytical Chemistry. New York. Holt, Rinehart and Winston. 1963. p. 458.

22. Fisher, R.B. and D.G. Peters. Basic Theory and Practice of Quantitative Chemical Analysis. London. W.B. Saunders Company, 3rd edn. 1968. p. 568.

23. Boyd A.W., C. Willis and R. Cyr. New Determination of Stoichiometry of the Iodometric Method for Ozone Analysis at pH 7.0. Anal. Chem. 42:670-672.

24. Alder, M.G. and G.R. Hill. The Kinetics and Mechanism of Hydroxide Ion Catalyzed Ozone Decomposition in Aqueous Solution. J. Amer. Chem. Soc. 72:1884-1886, 1950.

25. Stumm, W. Der Zerfall von Ozon in Wässriger Lösung. Helv. Chim. Acta. 37:773-778, 1954

SECTION XII

INACTIVATION KINETICS OF VIRUSES AND BACTERIA IN WATER BY OZONE

INTRODUCTION

As long ago as the end of the last century ozone (O_3) was suggested as a disinfectant for drinking water. Today, this chemical is being used to disinfect water supplies in many European countries. A review of the literature[1] suggests that in many ways ozone is superior to other chemical disinfectants: it acts quicker and in lower concentrations under certain conditions, and has few known side effects such as taste, odor and toxic by-products which are characteristic for some other chemicals e.g., chlorine.[2] These positive qualities have led to a renewed interest in ozone as a disinfectant of water for the inactivation of viruses which, under certain circumstances, appear resistant to chlorine.

It is striking that relatively little basic research has been done on the inactivation kinetics of microorganisms by O_3 at constant concentrations. In particular, the research with viruses has been neglected. Such research is important in order to understand the time-concentration relations associated with the killing power of the disinfectant, and would make it possible to calculate the quantities of ozone needed for different kinds of water.

In this study efforts were made to develop methods by which the disinfection abilities of ozone at constant levels of concentration can be tested. Various microorganisms found in polluted water were chosen. These included E. coli coliphage T_2, and poliovirus type I. The main thrust of the research was with viruses, since these are known to be more resistant to disinfectants than bacteria.

MATERIALS AND METHODS

Microorganisms

Poliovirus stock -

The same batch of poliovirus type I (Brunhilda) was used throughout this study. The virus was grown in Vero cells (Flow Laboratories), accumulated, and concentrated by the phase separation method,[3] and by two subsequent purification cycles of the concentrated virus. A cycle consisted of centrifugation of the suspension for 30 min at 12,000 x g. The supernatant was then centrifuged for one hour at 100,000 x g. The pellet was resuspended in 27 ml 0.005 M phosphate buffer, pH 7.0, and ultrasonicated (20 Mc/sec) for 2 min. Finally, the purified virus suspension was centrifuged at 4,000 x g for 30 min and stored in glass vials (0.25 ml) at -70°C. Titration of the virus was done on Vero cells, which indicated a count of 5.0×10^8 pfu/ml.

Coliphage T_2 -

Coliphage T_2 was grown on E. coli. The bacteria were inoculated in an Ehrlenmeyer flask containing nutrient broth, incubated in a shaker bath at 37^0C for 5 hours. Subsequently, 10^9 pfu bacteriophage were added and the suspension was incubated for an additional 3 hours. At the end of the entire incubation period (8 hrs) the suspension became almost clear. This suspension was centrifuged for 45 min at 12,000 x g; the supernatant was centrifuged for an additional 3-4 hours at 40,000 x g. The sediment was resuspended in phosphate buffer, pH 7.5, and again centrifuged. The cycle was repeated 4 times. After the last washing, the sediment was resuspended in phosphate buffer, diluted to 5 x 10^8 pfu/ml and kept under refrigeration.

E. coli (ATCC 11229)-

Before each experiment the bacteria were inoculated into 30 ml nutrient broth and incubated overnight at room temperature, followed by 4-5 hrs in a shaker bath at 37^0C. The bacteria were harvested and washed 4 times by centrifugation in 50 ml 0.5 M phosphate buffer (pH 7.0). The final sediment was resuspended in 50 ml of the same buffer solution. The suspension was then filtered through a Millipore filter (3.0μ) to remove possible clumps. The resulting solution usually contained 2 x 10^9 bacteria/ml.

Ozone

The ozone was generated from the oxygen in the air by means of a Fisher-Lab Ozonizer (OZ III). A suction pump (Charles Austen Pumps, Ltd.) was used to pass the air through the ozonizer. The air stream was dried by means of a Foty "Senior" air purifier and flow equalizer. The ozone-rich air was bubbled through a 0.05 M phosphate buffer, pH 7.0, until a high concentration of O_3 suspension was reached. The

O_3 excess remaining in the air stream was neutralized by passing the air stream through two traps, the first of which contained a concentrated solution of KI and the second, thiosulfate. Before each experiment, the concentrated solution was diluted until the appropriate ozone concentration was obtained.

Ozone concentration determination -

This was done by utilizing a spectrophotometric method developed in our laboratory described in Section XI.[4] Two similar procedures were developed: procedure "A" for low ozone concentrations (0.01-0.30 ppm) and procedure "B" for high concentrations (0.30-2.0 ppm).

In procedure "A", 10 ml of the ozone containing samples were introduced into a test tube containing 2 ml of 5% neutral potassium iodide buffered reagent. The test tube was left for about 30 min in a cool and dark place. Triiodide was quantitatively liberated and the intensity of absorbance was then read with a spectrophotometer (Zeiss P. MQ II, equipped with stoppered 20 and 40 mm quartz cuvettes, suitable for use in the U.V. region) using cells of 40 mm light path, against a distilled water blank.

In procedure "B" for higher ozone levels, 5 ml of the sample were introduced in a test tube containing 5 ml of 2% neutral potassium iodide. After about 30 min the intensity of the absorption was read using cells of 20 mm light path.

Ozone-demand-free water -

Water used as dilutant in the ozone experiments was made ozone-demand-free by distillation in an all-glass still in the presence of alkaline potassium-permanganate. This was followed by ozonizing the bidistilled water and dissipation of the ozone by boiling.

Ozone-demand-free glassware -

In the course of this study it was found that ozone-demand-free glassware is a prerequisite for maintaining a constant level of ozone concentrations during the experiments. All glassware was therefore cleaned with dichromate-concentrate sulphuric acid mixture, after which the glassware was rinsed in tap water and distilled water, soaked in a strong ozone solution and finally dried at 180°C.

Inactivation of poliovirus

One ml of virus stock suspension diluted 1:10 in ozone-demand-free water was put into a beaker containing 400 ml phsophate buffer (0.05 M, pH 7.2) and dissolved ozone in the desired concentration. Before and during the experiment the beakers were kept at 5°C ± 0.1°C or 1°C ± 0.1°C in a thermostatically controlled water bath. The contents of the beakers were constantly stirred at 80 rpm by glass paddles connected to a mechanical stirrer (Phipps and Bird). The beakers were covered to prevent the escape of ozone. Samples for virus determination were withdrawn by pipettes through orifices in the covers. The first sample (5 ml) was taken 8 seconds after the introduction of the virus. Further samples (5 ml each) were taken at 8-second intervals for the first two minutes, followed by sampling a 3, 5, 10 and 15 minutes. The samples were introduced into a 5 ml neutralizer (solution of 0.002 M tetra sodium pyrophosphate and 0.0004 M sodium sulphite) immediately upon withdrawal. "Sampling time" was recorded as the very moment the sample was blown from the pipette into the neutralizer. Pipetting never took more than 2 seconds at the most. An additional beaker containing 200 ml phosphate buffer and 0.5 ml of the diluted virus stock (without ozone) was stirred simultaneously in the same water bath to act as control. Samples for ozone determination were drawn immediately before and 1, 2, 5, 10 and 15 min after virus addition. Neutralized samples were assayed virus in tissue cultures on the day of the experiment. The preparation of dilution and inoculation into tissue cultures have been described elsewhere.[5]

In order to decrease the sampling time to less than 8 seconds, a simple fast-mixing apparatus was set up. This apparatus consisted of two 10 ml glass syringes (one containing ozone solution and the other the virus suspension) which could be injected simultaneously into a mixing device. The two injection parts of the mixing device were of 4mm i.d. and led into a 2 mm diameter mixing chamber, the resulting mixed solution being forced out at right angles through a 2 mm tube. The entire mixing assembly was made of Teflon. With the present simple set-up, which was operated manually, it was possible to sample after reaction times of about half a second. The reaction being quenched by flowing into the neutralizing sulfite solution. Longer reaction times could be obtained by increasing the length travelled by the mixed solution before being quenched. While the above apparatus and the manual operation are obviously not capable of accurate reproducibility it represents a simple, quick and inexpensive way to examine the profitability of virus inactivation studies in the one-half to three second range.

Inactivation of coliphage T_2

The procedures followed here were as with poliovirus, with the exception of the sampling frequency, which was done every 10 seconds. For assays of bacteriophages, samples were diluted in nutrient broth. One ml of each dilution was mixed with 1 ml 1.5% liquified agar (52°C) containing E.coli bacteria. After stirring, the mixture was poured onto petri dishes containing agar. Plaques were counted after 18 hr incubation at 37°C.

Inactivation of E. coli

The experiments were essentially the same as with coliphage T_2. Bacterial assays were done by the pour plate method in nutrient agar.

Redox Potentials

These were measured with a potentiometer (Radiometer Type PHM 26C) with calomel- and platinum electrodes. The potentials are given as the uncorrected values in millivolts, using the calomel electrode as reference cell.

RESULTS

Inactivation Kinetics of Poliovirus 1 by Ozone

Concentrations of 0.07, 0.1, 0.15, 0.2, 0.3, 0.5, 0.8, 1.5 and 2.5 ppm ozone were used. Figures 17, 18 and 19 depict the inactivation

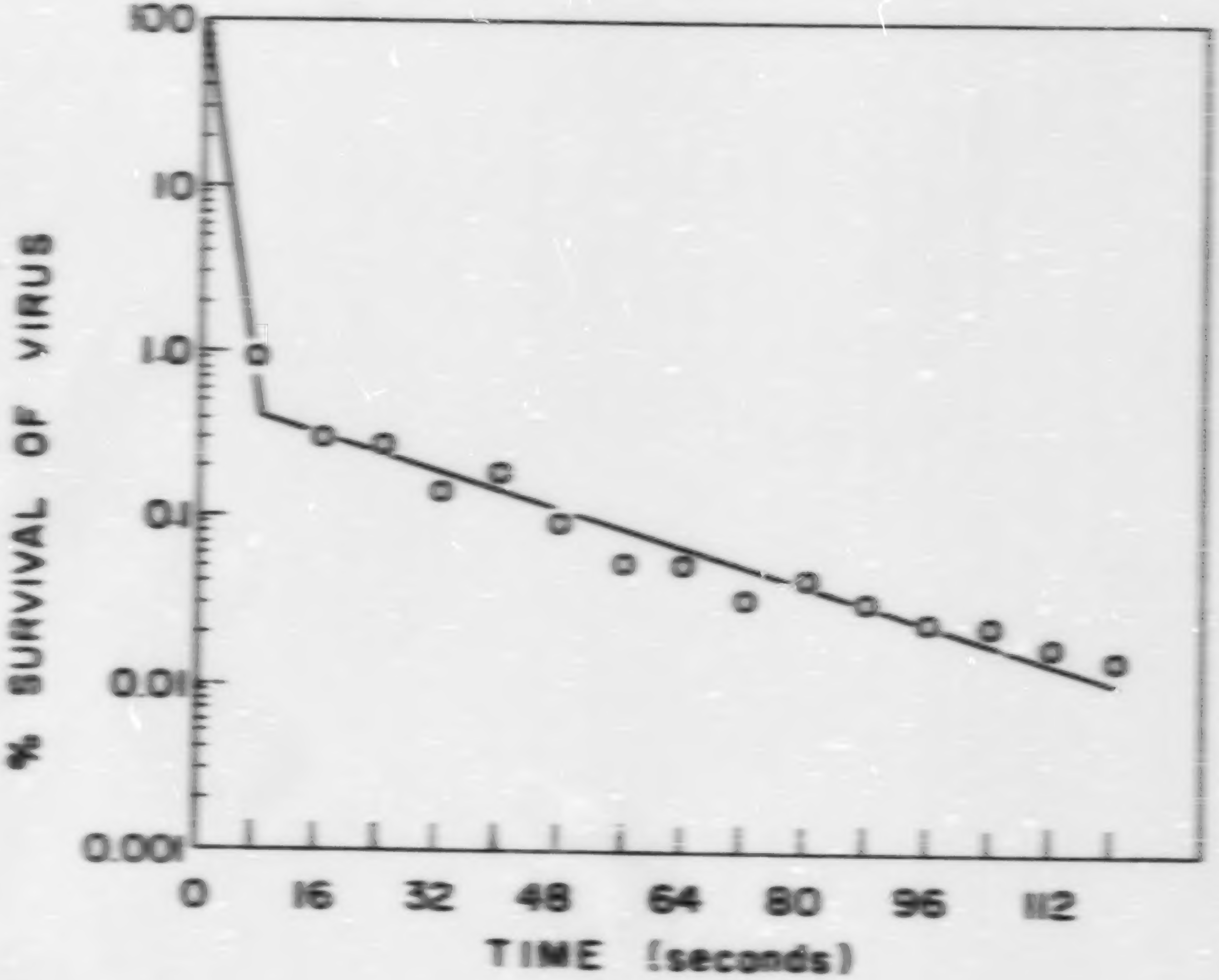


Fig. 17. Inactivation kinetics of poliovirus 1 by 0.3 ppm Ozone at 5°C.

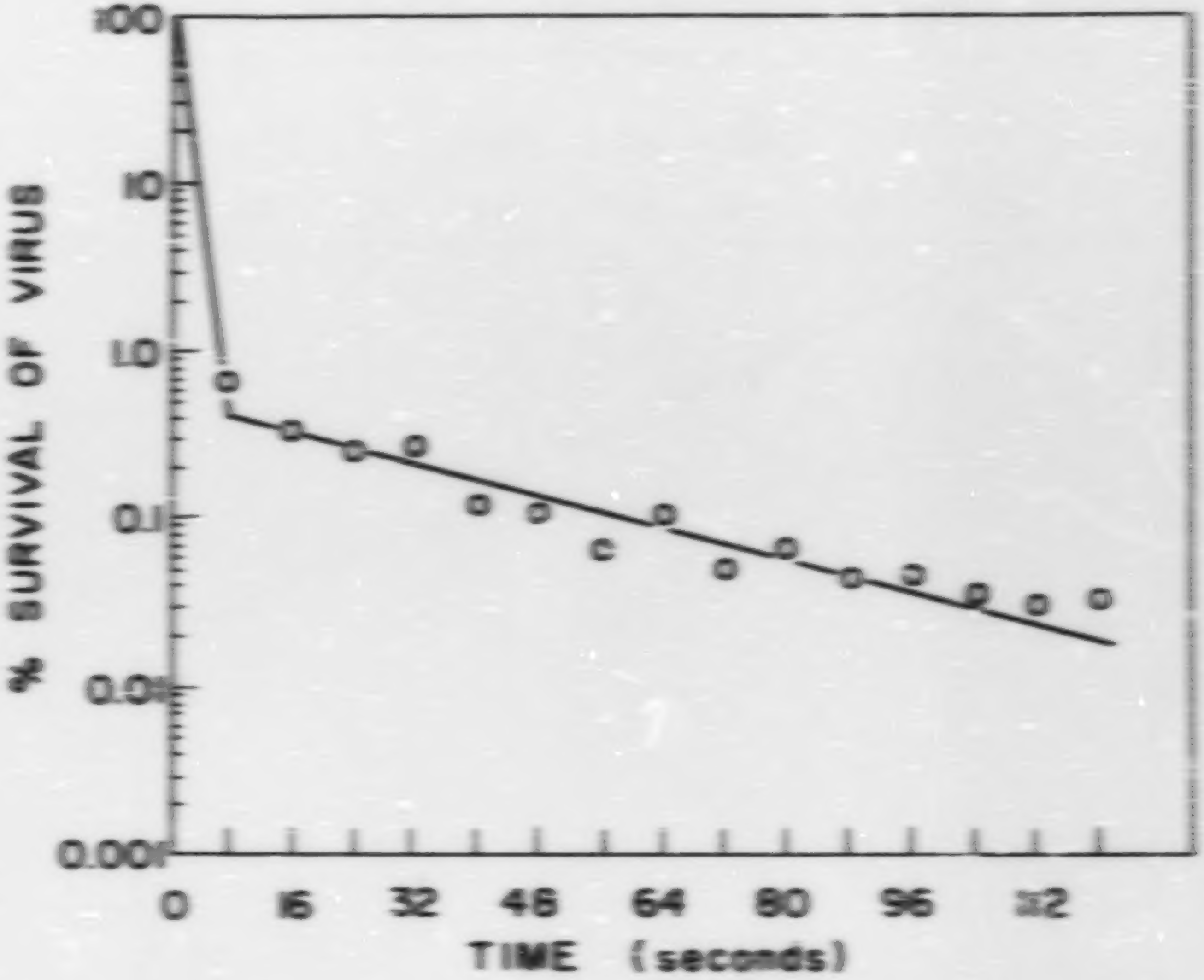


Fig. 18. Inactivation kinetics of poliovirus I by 0.8 ppm Ozone at 5°C.

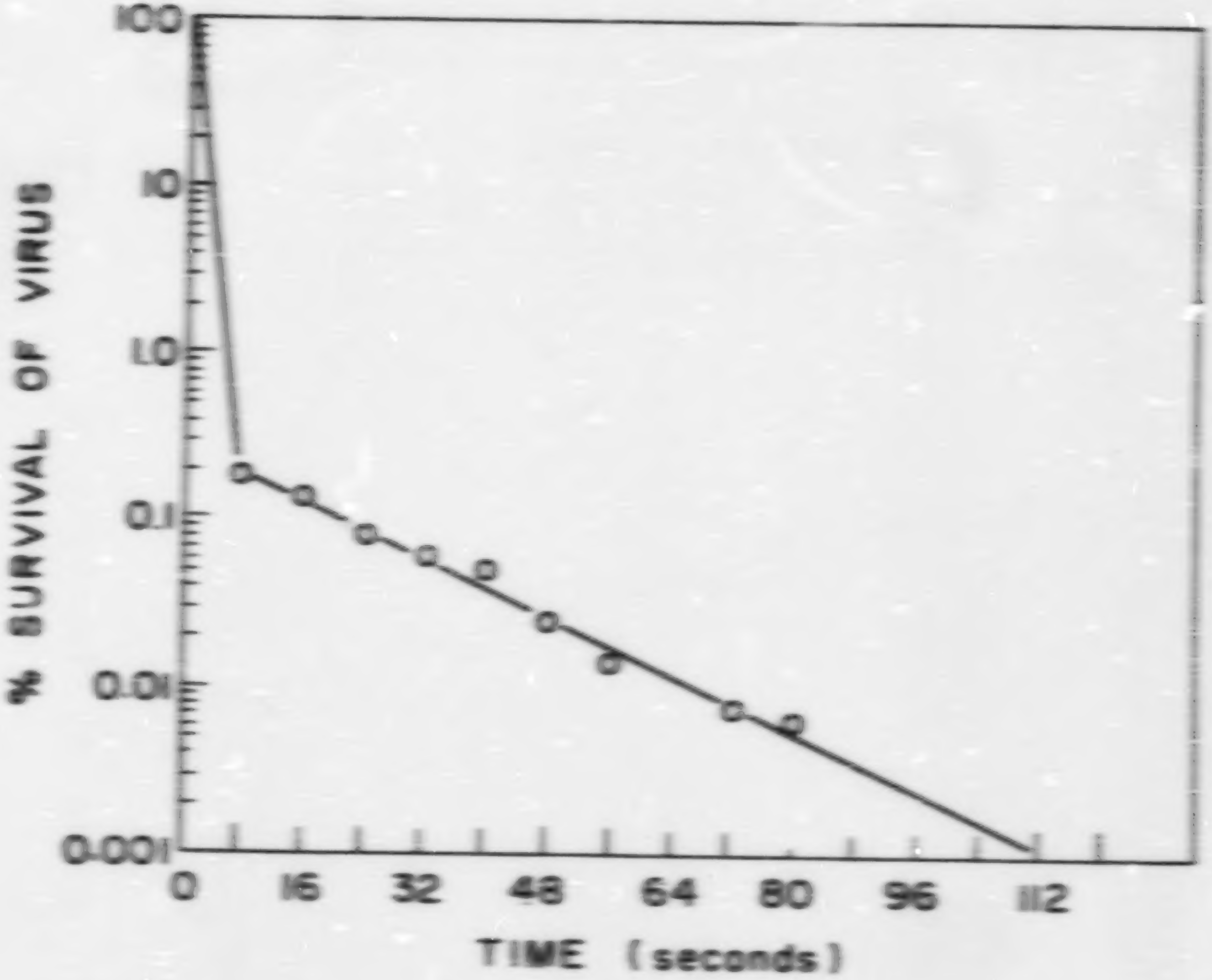


Fig. 19. Inactivation kinetics of poliovirus I by 1.5 ppm Ozone at 5°C.

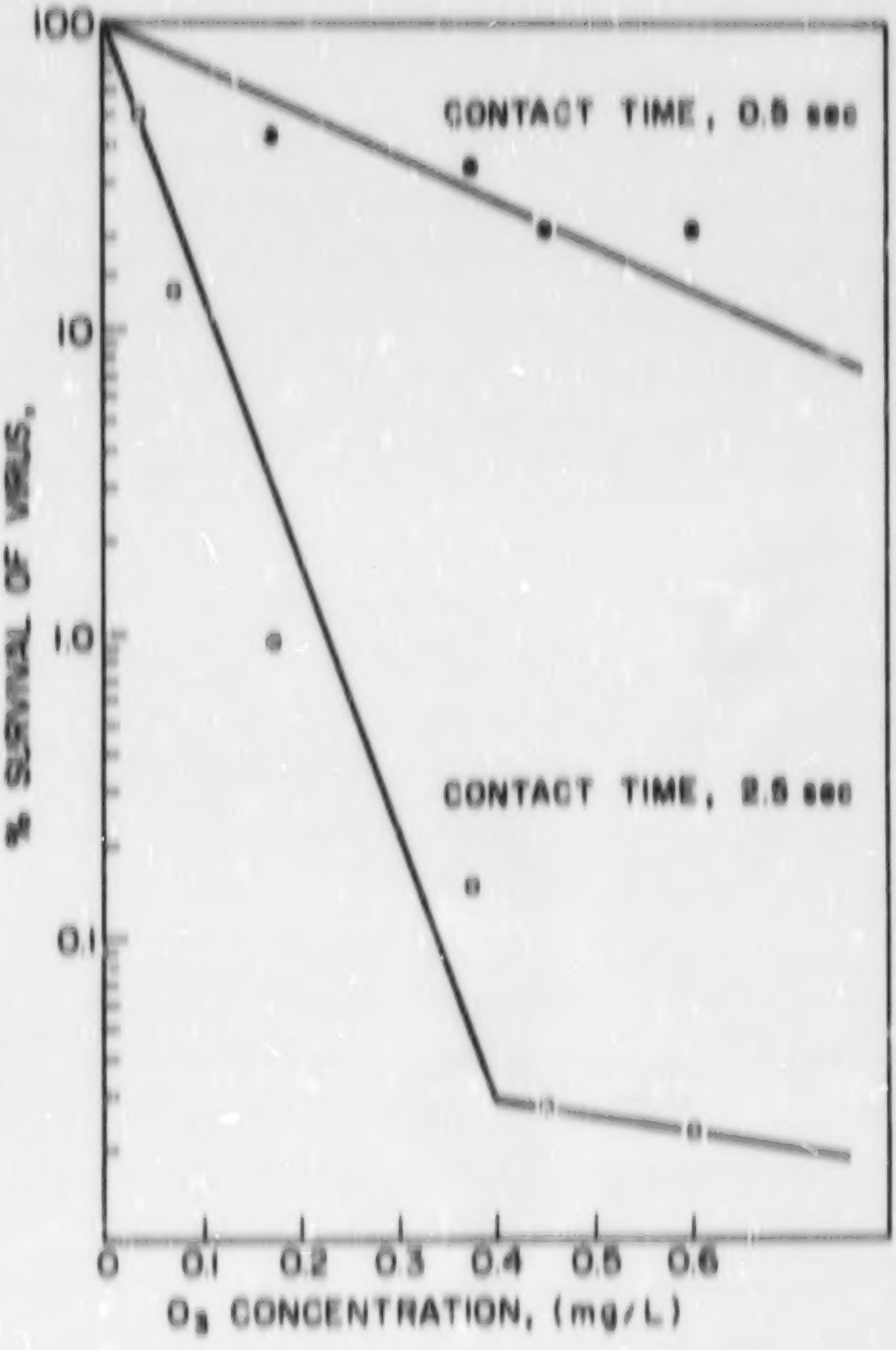


Fig. 20. Virus survival rate with varying Ozone concentrations at 0.5 sec and 2.5 sec

kinetics of the viruses with ozune concentrations ranging from 0.3-1.5 ppm at 5^0C. Each graph sums up 3 experiments at least. A significant feature is the two-stage action of ozone. The first stage is short, less than 8 seconds, with a virus kill of 99-99.5%. Stage 2 lasts from 1 to 5 min, and in this period the remaining viruses are inactivated. Increasing the ozone concentration from 0.2 ppm to 1.0 ppm had very little effect on the inactivation rate, while at ozone concentration of 1.5 ppm and above there was a somewhat higher rate of inactivation during the second stage.

What appears to be a true dose response is, however, seen in Fig. 20. In this preliminary experiment, the effect of several concentrations of O_3 on poliovirus I, during very short periuds of time (0.5 and 2.5 seconds) was measureu. The results clearly show a higher percent kill with the increased O_3 concentrations. The effect is already noticeable after 0.5 second of contact and is very strong after 2.5 seconds.

During the initial phase of this study, it was found that ozone concentrations lower than 0.15 ppm did not appear to cause virus inactivation, as shown in Figure 21 which depicts the percent survival of poliovirus type I after 40 seconds of exposure to various concentrations of ozone. In later experiments, inactivation was obtained with 0.1 and 0.05 ppm ozone with the same virus stock. There was, however, a lack of consistency in these results and in some of the experiments no inactivation was seen.

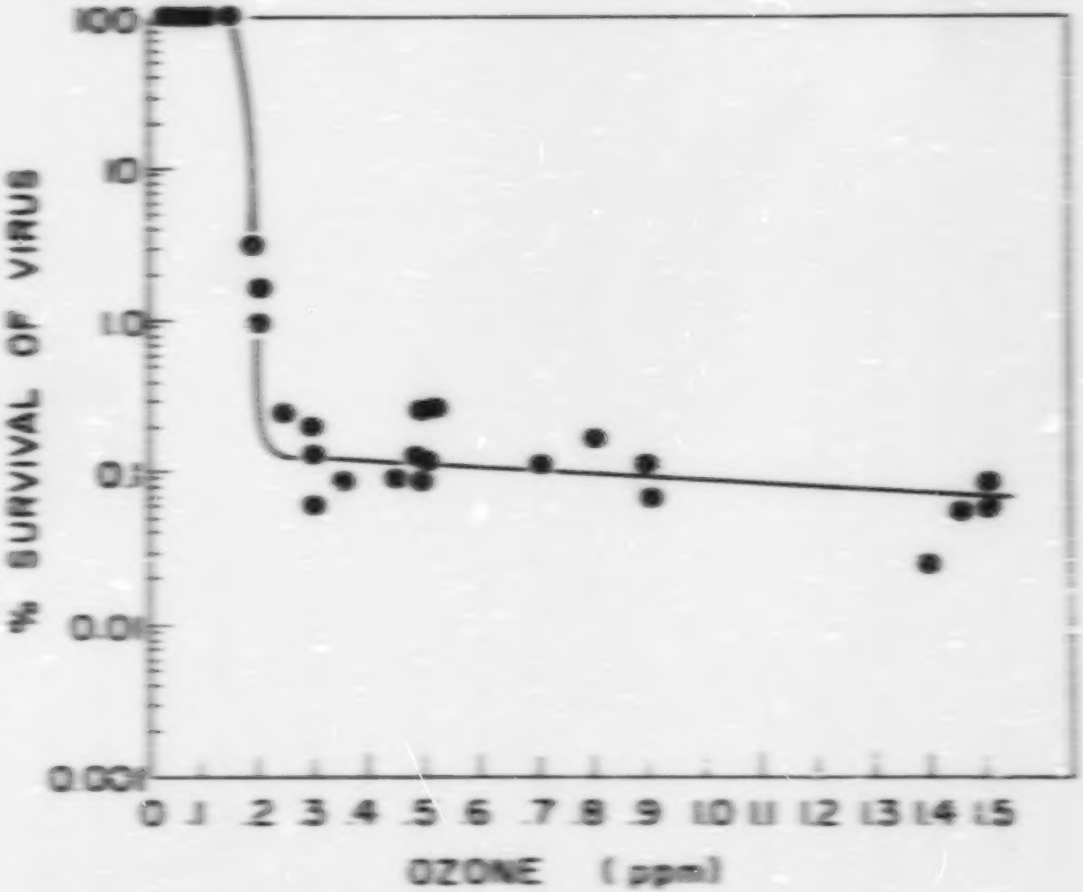


Fig. [illegible]. [illegible] survival after [illegible] of exposure to various concentrations of Ozone

Effect of Ultrasonic Treatment

Berg et al[6] have suggested formation of virus clumps as a possible explanation for the differences in sensitivity of the virus stocks to chemical disinfectants. Such virus clumps can be expected to be more resistant to low concentrations of disinfectants. Ultrasonication causes the break-down of clumps of microorganisms. A preparation of poliovirus was therefore ultrasonicated for 2 min at 20 mc/sec (M.S.E. 100 watt) and the ultrasonicated stock was then tested in inactivation experiments with 0.1 ppm O_3 (Fig. 22). The effect of the ultrasonic treatment may be said to have been dramatic. While before the treatment the initial kill (after 8 seconds contact) was less than 90% (and even after contact of 15 minutes 0.2-0.5% of the virus could still be found), after ultrasonication the virus became extremely sensitive to the same O_3 concentration. This found its expression in an initial kill (after 8 seconds) of 99.5% and in a complete disappearance of the virus after 3 minutes i.e., a decrease of more than 99.999%. The effect of ultrasonication alone on the virus titer of the stock was negligible.

Effect of Storage Temperature

Ultrasonication experiments provided a possible explanation for the fluctuation in virus sensitivity in O_3, but it did not explain how and when these shifts occur. Certain circumstantial evidence, which became apparent on careful reconstruction of the records concerning the storage of the virus stock, helped throw some light on this question.

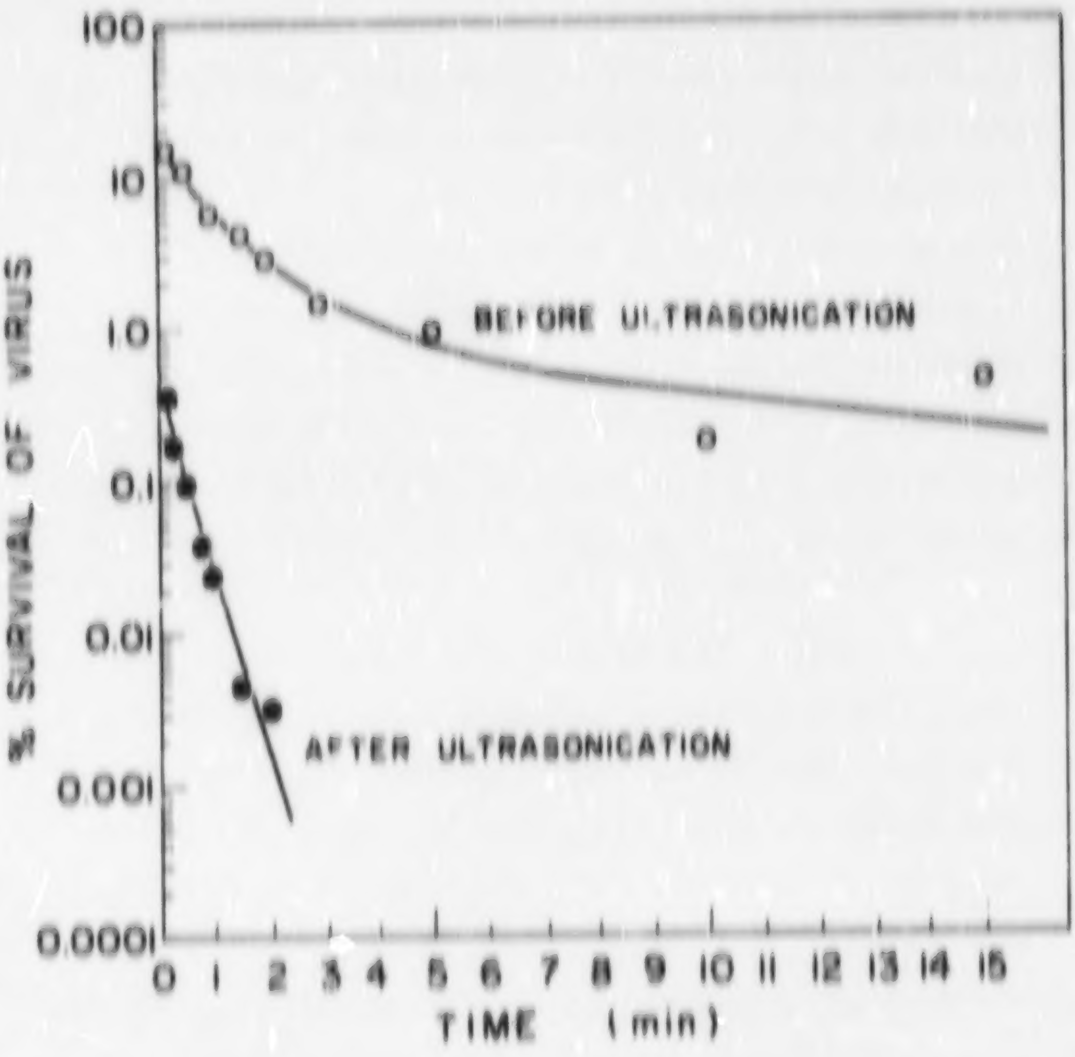


Fig. 22. Inactivation kinetics of poliovirus 1 (118-h), before and after ultrasonication, by 0.1 ppm Ozone

For technical reasons the virus stock had to be transferred from its original -70°C storage to -15°C for a period of several weeks. The records indicated that only after this change did this particular batch show an increased resistance to 0.1 ppm O_3. This observation led to the question whether storage temperature affects poliovirus sensitivity to O_3. In trying to find the answer, the following experiment was carried out: 10 ml of virus stock solution were ultrasonicated for 2 min and divided into ampoules of 0.25 ml each (a quantity sufficient for one inactivation experiment). Half of the ampoules were then stored at -70°C and half at -15°C. Samples were removed at different time internals from both freezers and inactivation kinetics experiments with 0.1 O_3 were carried out in the usual manner.

The virus stored at -70°C revealed a high sensitivity to O_3 treatment, identical to the reaction of the freshly prepared and ultrasonicated virus batch (Fig. 22). Its sensitivity did not undergo any change even after storage of 2 months. In contradistinction, the virus batch stored at -15°C showed a drastic change in sensitivity after only 5 days of storage. This change expressed itself in the virus returning to a resistant inactivation pattern similar to that seen before ultrasonication. In further experiments virus stock from the -15°C freezer was ultrasonicated for a second time and became sensitive again. Transferring the sensitive ultrasonicated virus from -70°C to -15°C resulted in a shift to the resistant form, a reaction similar to that shown by untreated virus.

Inactivation Kinetics of Coliphage T_2

O_3 concentrations ranging from 0.01-1.3 ppm were used. The inactivation kinetics of coliphage T_2 with 0.01, 0.09 and 0.26 ppm O_3 at 1°C are depicted in Figure 23. Similar results were obtained at 5°C. As with poliovirus, both the two-stage inactivation curve and the lack of a clear dose-response above concentrations of 0.2 ppm O_3 are seen. Lower concentrations caused a limited "dose response" occurring in the first stage only, and was expressed in an initial kill of (A) 99-99.9% at O_3 concentrations ranging between 0.01-0.06 ppm; (B) kill of 99.9-99.99% at 0.07-0.19 ppm O_3; (C) kill of 99.9-99.999% at 0.2 ppm O_3.

Inactivation Kinetics of E.coli

The O_3 concentrations ranged from 0.02-1.55 ppm. Figure 24 shows the inactivation kinetics at 1°C. Similar results were obtained at 5°C. Again, the two-stage inactivation curve is seen. Above 0.1 ppm O_3 no dose response was obtained. The lowest concentration causing inactivation was 0.04 ppm O_3. Some sort of "dose response" could be seen when the concentrations ranged between 0.44-0.07 ppm O_3.

Introduction of Virus at Two Different Times

A possible explanation for the two-stage inactivation curve could be provided by a chemical change of ozone e.g., the appearance of some intermediate decomposition species, triggered by the microorganisms

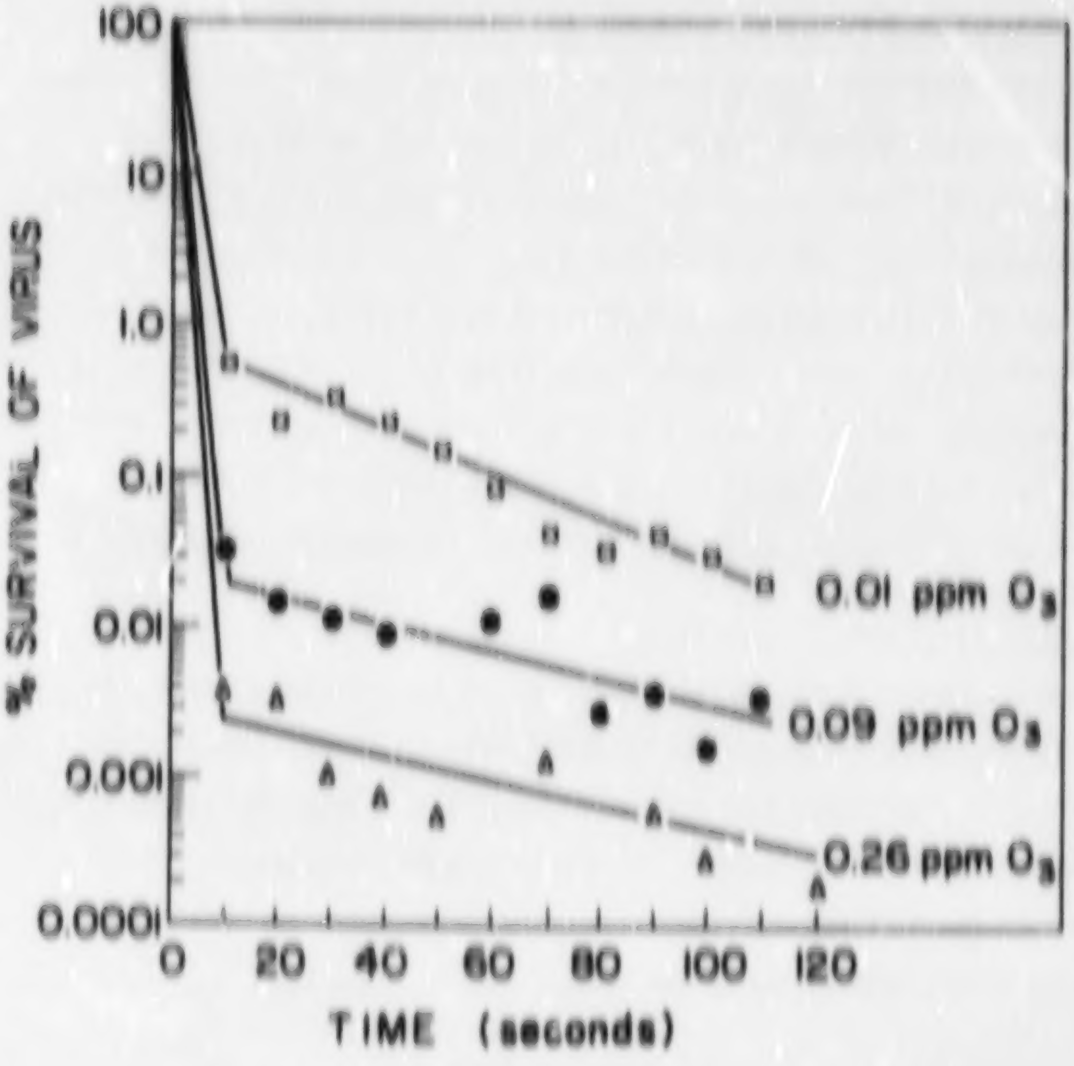


Fig 77. Inactivation kinetics of coliphage f_2 by var concentrations of Ozone at 1°C

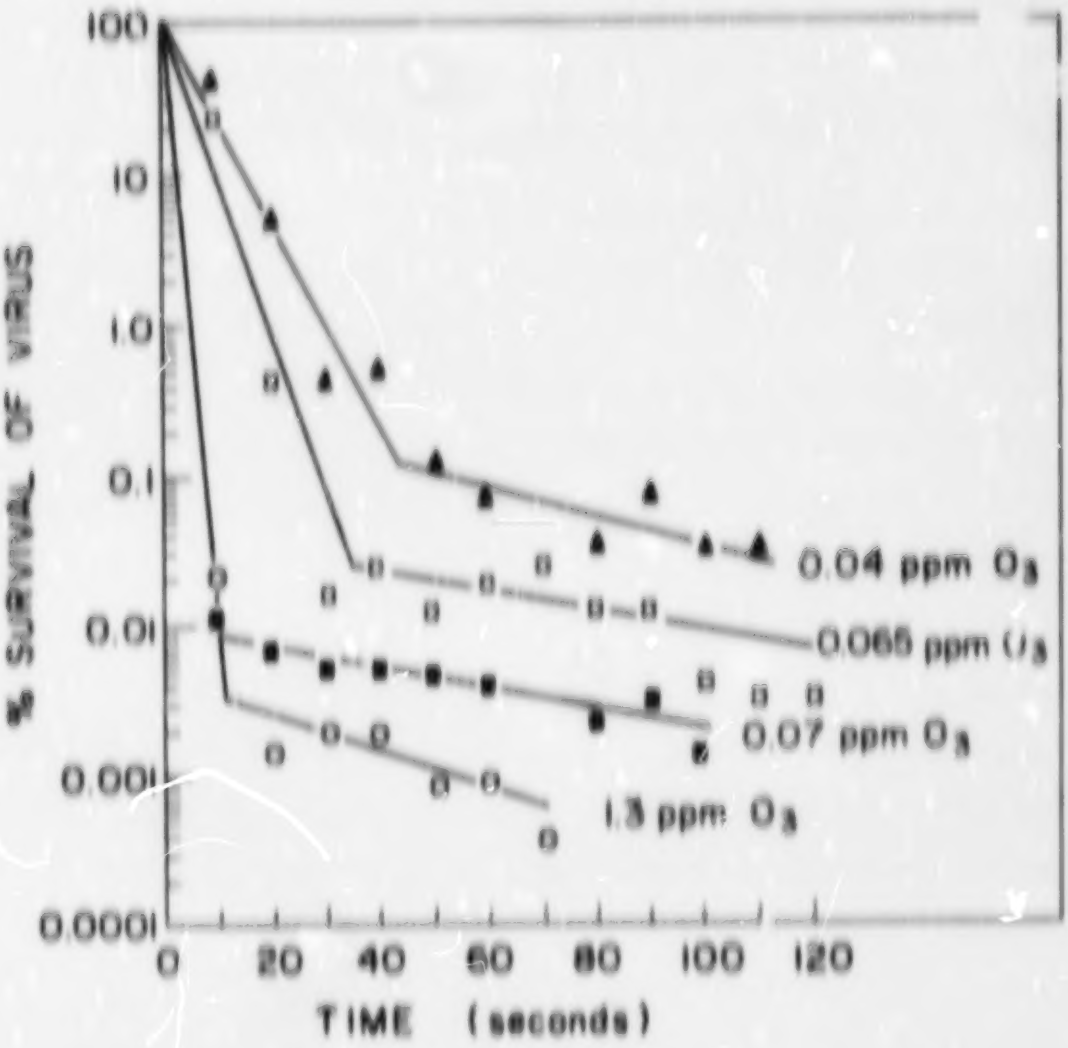


Fig. 26. Inactivation kinetics of E. coli by various concentrations of Ozone at 1°C

or organic contaminants introduced with the virus stock. A second portion of poliovirus was therefore added 56 seconds after the first administration. The inactivation rate of this additional virus inoculum was the same as of the first portion, which implies that the inactivating power of the ozone, whose measured concentration did not change, was not affected by the experimental conditions (Fig.25).

Redox Potentials of Ozone Solutions

To verify a possible connection between the redox potentials and the disinfection ability of ozone,the redox potentials of different O_3 concentrations were measured. Figure 26 shows the results of many such measurements. Redox potentials increase rapidly with increasing O_3 concentrations from 0.03-0.2 ppm, after which there is a levelling off.

DISCUSSION AND CONCLUSIONS

During the search for disinfectants of bacterial contamination in water, ozone was one of the first chemical agents found to possess germicidal properties.[1] However, its mode of action, particularly against viruses, has been investigated less than that of other disinfection agents. Since there is a dearth of data on its mode of action, it was decided to study the behavior of ozone in clean water. Ozone is extremely labile; even a very small amount of organic matter in the water causes its rapid dissipation. Extreme care was required to build an experimental system in which ozone loss could be kept at a minimum. Toward this purpose, ozone-demand-free glassware, microorganism

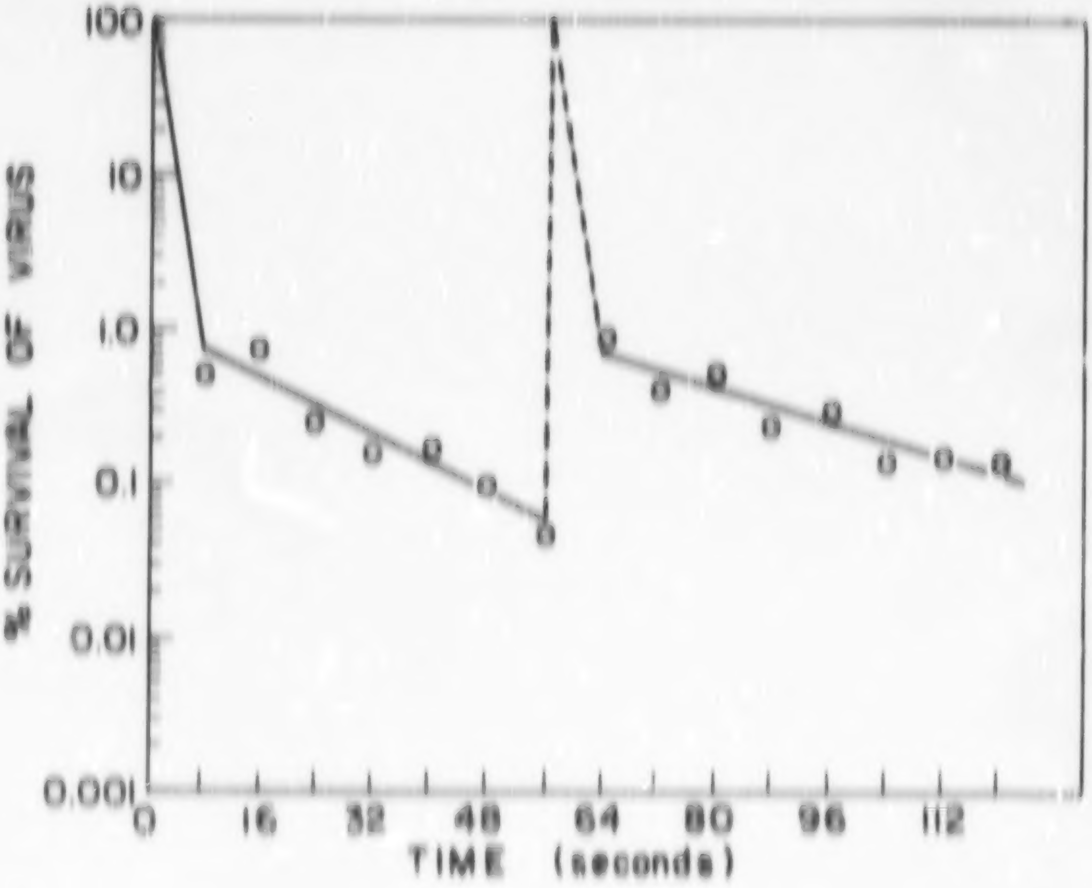


Fig. 25. Inactivation kinetics of poliovirus 1, added in two portions, at 0 time and after 56", by 1.5 ppm Ozone

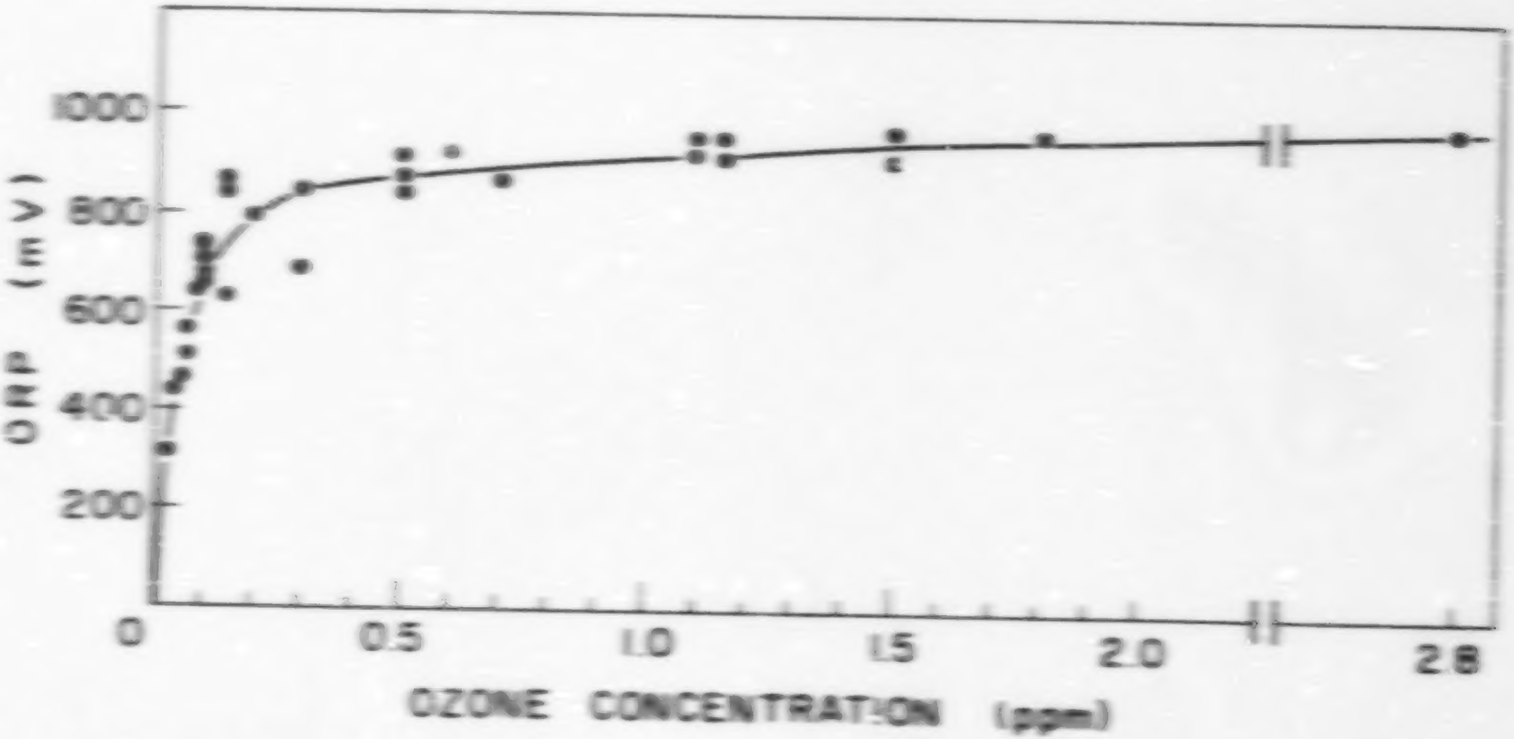


Fig. 35. Correlation between Ozone concentration and redox potential values

stock and water had to be prepared. Further measures were covering the beakers, maintaining the temperature at 0°C or lower during the experiments, and stirring not exceeding 80 rpm. Stirring above 80 rpm leads to a rapid loss of ozone.[4] In spite of these meticulous preparations, it was not always possible to keep the ozone at a constant level. In this study results are reported only for those experiments where ozone dissipation did not exceed 20% during the 15-minute period of the experiment.

The main object of this work was to elucidate the inactivation kinetics of microorganisms in water. Since poliovirus type I is ubiquitous in sewage it was chosen as a prototype for viral contamination in water. Another virus indicator used in this work was the bacterial virus coliphage f_2. Finally, E. coli a bacterial indicator of fecal contamination of water was also included.

An outstanding characteristic of ozone action is its rapid inactivation of the microorganisms tested and the low concentrations of O_3 needed. Comparing the results obtained in this study with those of Scarpino et al,[7] who used chlorine, and with those of Berg et al,[8] who used iodine, the superiority of ozone is striking. With 0.3 ppm of disinfectant, for example, a 99% kill of poliovirus was reached in less than 10 seconds when using ozone as compared to approximately 100 seconds needed with chlorine (at pH 10) and about 100 minutes with iodine. In fact, ozone acts so quickly that it is practically impossible to measure the time required for 99% kill. It was not possible to obtain curves showing the inactivation rate as a first order reaction according to Chick's Laws.[8]

A noteworthy phenomenon was the two-stage shape of the inactivation curve. Stage 1 lasted less than 10 seconds with an inactivation of more than 99%. Stage 2 continued for several minutes during which period final inactivation occurred. This feature cannot be ascribed to changes in the composition of ozone, since poliovirus added 56 seconds after the first inoculum was inactivated at the same rate. A possible explanation for the two-stage inactivation kinetics might be that 0.5-1.0% of the poliovirus consists of clumps. Galasso and Sharp[9] were the first to mention such clumps, and Berg et al[6] described these virus clumps as impeding the inactivation rate. It is interesting to note that this two-stage action also appeared in experiments with coliphage T_2 and E.coli. It may be assumed that here, too, clumps were the cause of this phenomenon. An attempt was made to obliterate the clumps in the T_2 phage and E.coli cultures and, indeed, an accelerated rate of kill with these two microorganisms was seen during the first stage. O_3 concentrations as low as 0.01 ppm (the lowest concentration that could be measured) resulted in a rapid inactivation of the coliphage, while E.coli was inactivated by 0.04 ppm O_3.

Increasing the O_3 concentration to above 0.2 ppm had very little effect on the inactivation rate (as far as could be discerned by us).

Since, for technical reasons, the earliest sample could be taken only 8-10 seconds after the introduction of the microorganisms, it was impossible in these experiments to determine the processes that take place during 0-10 seconds. Dose response during this period may occur. To determine this, special apparatus for obtaining contact times of one to ten seconds would be required. A preliminary experiment tends to confirm this hypothesis, as is seen in Fig. 20. These results, however, were obtained with a crude method. Better equipment is now under development for future study.

With concentrations lower than 0.2 ppm O_3, the inactivation curves of poliovirus were not sufficiently reproducible to allow an accurate interpretation of the results. In some experiments these ozone concentrations had no effect, while in others, concentrations even lower than 0.05 ppm caused rapid inactivation.

Berg et al[6] maintain that clumping affects virus resistance. Ultrasonication (said to disintegrate the clumps) of a relatively resistant batch of poliovirus I indeed resulted in an increased sensitivity to 0.1 ppm O_3. It is interesting that, while the sensitivity of the virus to ozone increased, the titer did not change although it would be reasonable to expect an increase in the titer after disintegration of the clumps. This may be explained by a simple calculation. Let us assume that 5% of the virus was in the form of clumps, and that an average clump consists of 10 virions. Even the complete disintegration of all clumps would thus result in an increase of about 50% in virus number, an increase not considered to be significant by the usual methods of virus quantitation. A pronounced increase in virus sensitivity, however, would be shown by the techniques used in the present study.

The phenomenon of sensitive virus becoming resistant after having been stored in a freezer still remained unexplained. There was reason to suspect that the storage temperature may have been the causative factor. To test this assumption, a series of experiments were performed in the course of which it became apparent that storage at -15^{0}C is inadequate for poliovirus I in inactivation experiments with O_3. This inadequacy expressed itself in transformation of the virus from sensitive to resistant after only 3 days at -15^{0}C. On the other hand, storing for 2 months at -70^{0}C did not cause the slightest change in sensitivity of the virus. It should be remarked that samples transferred from -70^{0}C to -15^{0}C--without thawing--also shifted to the resistant form, which might well explain how our poliovirus changed its level of sensitivity.

The most acceptable explanation for the virus stock shifting from sensitive to resistant appears to be that a significant percentage of the viruses form clumps. However, other possible physical changes resulting from storage of virus at various subfreezing temperatures cannot be ruled out at this stage.

An association may exist between the mode of action of O_3 and its redox potential.[10] Redox potentials increased with increasing concentrations of ozone from 0.03 to 0.2 ppm, but about 0.2 ppm O_3 the potentials levelled off. The fact that only a minor increase in redox potentials was seen above 0.2 ppm O_3 correlates well with the lack of dose response in the inactivation curves. However, redox potential values do not explain our results of ozone action on the microorganisms with concentrations lower than 0.2 ppm O_3.

Lowering the temperature from 5°C to 1°C had nearly no effect on the inactivation kinetics of the microorganisms, contrary to chlorine. It should be noted that O_3 is chemically very active, even at temperatures as low as -70°C.

The work described here should be regarded as preliminary. Special techniques are being developed which will enable us to measure the inactivation rate of microorganisms by ozone during the first 10 seconds (first stage). For research on water disinfection, however, these findings are important since they clearly show it to be imperative to store the virus, used in the experiments, under uniform conditions at -70°C if changes in sensitivity during the course of the experiments are to be avoided.

The major unanswered question is, however, whether viruses in the natural aqueous environment are of the sensitive type or whether they include a significant percentage of clumps, thus possibly following the resistant pattern of inactivation under practical field conditions of water and wastewater disinfection. Our plans now call for a further investigation of this critical point as well as studies using ozone in contaminated water and effluent. But, whatever remains to be done, the rapidity with which ozone acts at low concentrations, as was demonstrated here, promises positive utilization of ozone in the disinfection of water and wastewater.

REFERENCES

1. Venosa, A.D. Ozone as a Water and Wastewater Disinfectant. In: Ozone in Water and Wastewater Treatment. Evans, F.L. (ed.). Ann Arbor, Science Publications, 1972. p. 83-100.

2. Berg, G. An Integrated Approach to the Problem of Viruses in Water. In: Proc. Natl. Specialty Conf. on Disinfection, Amer. Soc. Civil Engineers Publication, 1970. p. 339-364.

3. Shuval, H.I., B. Fattal, S. Cymbalista, and N. Goldblum. The Phase-Separation Method for the Concentration and Detection of Viruses in Water. Water Res., 3:225-240, 1973.

4. Shechter, H. Spectrophotometric Method for Determination of Ozone in Aqeous Solutions. Water Res., 7:729-739, 1973.

5. Shuval, H.I., A. Thompson, B. Fattal, S. Cymbalista, and Y. Weiner. Natural Virus Inactivation Processes in Seawater. J. Sanitary Eng. 97:587-600, 1971.

6. Berg, G., S.L. Chang, and E.K. Harris. Devitalization of Micro-Organisms by Iodine. Virology 22:469-481, 1964.

7. Scarpino, P.V., G. Berg, S.L. Chang, D. Dahling, and M. Lucas. A Comparative Study of Inactivation of Viruses in Water by Chlorine. Water Res., 6:959-965, 1972.

8. Chick, H. An Investigation of the Laws of Disinfection. J. Hyg. 8:92-158, 1908.

9. Galasso, G.L. and D.G. Sharp. Virus Particle Aggregation and the Plaque Forming Unit. J. Immunol. 88:339-347, 1962.

10. Chang, S.L. Modern Concept of Disinfection. In: Proc. Natl. Specialty Conf. on Disinfection, Amer. Soc. Civil Engineers Publication, 1970. p. 635-680.

SECTION XIII

DISINFECTION OF VIRUSES IN SEWAGE BY OZONE

INTRODUCTION

In a previous study describing ozone (O_3) action on microorganisms in ozone-demand-free water,[1] it was found that low concentrations of O_3 rapidly inactivate these entities. Compared to chlorine, which is widely used for disinfection of water, O_3 acts ten times as fast. The above study was performed under optimal conditions, using water and glassware that were ozone-demand-free. Such situations certainly do not exist in the "true-world". The different kinds of water that have to be disinfected (drinking water, surface water, renovated water, or treated sewage effluents) at all times contain a certain amount of organic matter which reacts with the O_3 thus causing a decrease in O_3 concentration thereby creating ozone demand. A number of articles,[2,3,4] indeed point out that the O_3 concentrations required to inactivate viruses in water possessing O_3 demand, are considerably higher than concentrations needed under optimal conditions.

In the present study, O_3 was tested for its ability to inactivate water borne viruses in the "true world". As a model, poliovirus type I was chosen, which was introduced into filtered sewage.

MATERIALS AND METHODS

Poliovirus Stock

The same batch of poliovirus Type I (Brunhilda) was used throughout the study. Preparation and titration of the virus stock has been described elsewhere.[1,5]

Ozone

Ozone was generated from the oxygen in the air, as described previously.[1]

Analysis for Ozone in the Presence of Sewage

The method used for determining O_3 in effluents was the same iodometric-spectroscopic technique as described for the clean water system,[6] but with the following modifications due to the changing color of the effluent solution under investigation. The original color of the effluent was a pale yellow, which on ozonization slowly disappeared. It was therefore not possible to use the original solution as a blank for spectroscopic measurement. Instead, together with each sample removed for analysis, a duplicate sample was taken and diluted with an identical buffered solution without potassium iodide, and this sample was then used as the reagent blank. With this modification it was possible to measure the O_3 concentration of the solution under investigation.

Ozonization Vessel

The ozonization experiments were performed in a 500 ml water jacketed vessel. A Teflon stop-cock near the bottom of the vessel allowed for the removal of samples for analysis. The virus and O_3 were introduced into the flask via a glass stoppered opening at the top of the vessel.

Continuous ozonization of the effluent was also possible by introducing a sintered glass tube through the glass stopper. This allowed the ozone-rich air to be emitted into the effluent solution in the form of finely divided bubbles.

Filtered Sewage

Twenty liters of raw domestic sewage were filtered through a fiberglass filter (Whatman GF/B) for removal of suspended solids. The clarified effluent, which had a BOD of 500 ppm, a COD of 700 ppm, total Nitrogen 38 ppm and Ammonia 40 ppm, was divided into plastic bags (200 ml) and frozen at -80°C. The bags were defrosted one at a time (one for each experiment) and the effluent was diluted with phosphate buffer (M 0.05, pH 7.0), according to the specific experimental requirements.

Inactivation Experiments

These were carried out in either of the following manners: (a) Batch experiments, in which 0.5 ml of concentrated virus (1.0×1.0^{8} pfu/ml) was introduced into 20 ml filtered sewage; the admixture was then rapidly added to 180 ml buffer containing a known concentration of O_3 which had been placed in the ozonization vessel. In this way, a 10% solution of effluent was formed. For a 5% solution, 0.5 ml of concentrated virus was added to 10 ml of effluent, which was then introduced into 190 ml of the buffer. Constant stirring by a magnetic stirrer was maintained throughout the experiment. Samples (5 ml) for virus titration and ozone residual determination were withdrawn at 10 sec, one min and 5 min after introduction of the virus-effluent mixture. The samples for virus determination were added to a reducing solution to stop the ozone activity.[1]

(b) Continuous ozone bubbling, in which 1 ml virus stock (1.0×10^8 pfu/ml) was added to 400 ml of undiluted filtered sewage in the ozonization vessel. Magnetic stirring was then started and continued throughout the experiment. Ozone-rich air at a rate of 0.5 L/min was bubbled through the effluent-virus admixture. Samples for virus titration and O_3 determination were withdrawn as in method (a), but at different time intervals: the first sample was taken prior to O_3 bubbling, the following samples (after O_3 bubbling had been started) were withdrawn at 15-second intervals up to two minutes; the tenth sample was taken at three minutes. The samples for virus determination were added to a reducing solution.[1] In both methods a constant temperature of 5°C was kept. Virus assay was done on the same day, according to Shuval et al.[5]

RESULTS

Batch Experiments

The interaction of 0.4, 0.6, 1.3 and 1.8 mg/L O_3 (initial concentration) and the virus in the diluted and filtered sewage was examined. Each of these O_3 concentrations was tested with 5% and 10% effluent in buffer. The most striking observation was the complete disappearence of O_3 immediately upon the addition of the virus-effluent admixture. At the same time, a sharp decrease (90-99.9%) in virus titer by most O_3 concentrations was seen (Figs. 27, 28, 29, 30). This virus

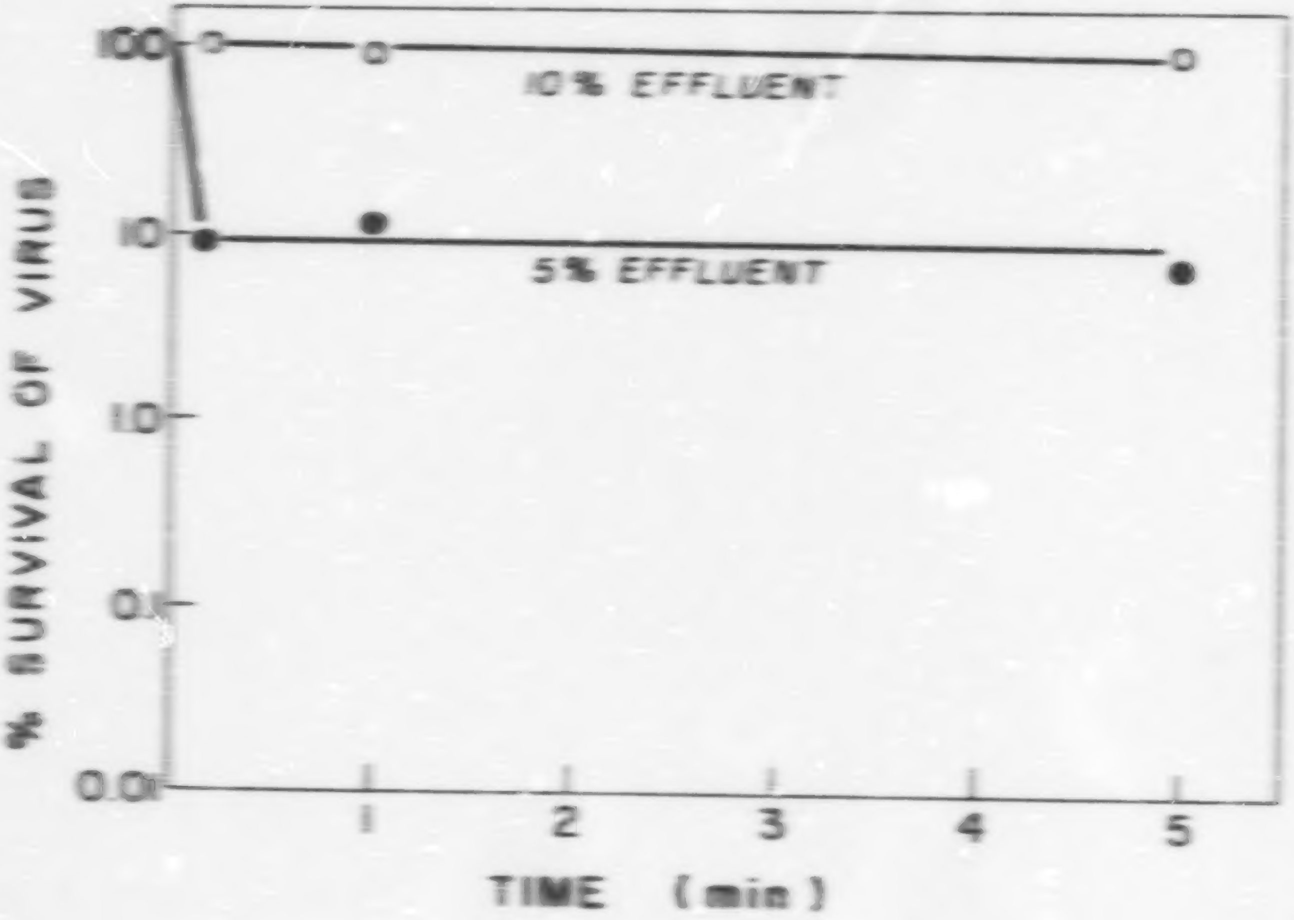


Fig. 27. Inactivation kinetics of poliovirus 1 by 0.4 mg/l initial O_3 concentration in the presence of 5% and 10% filtered sewage

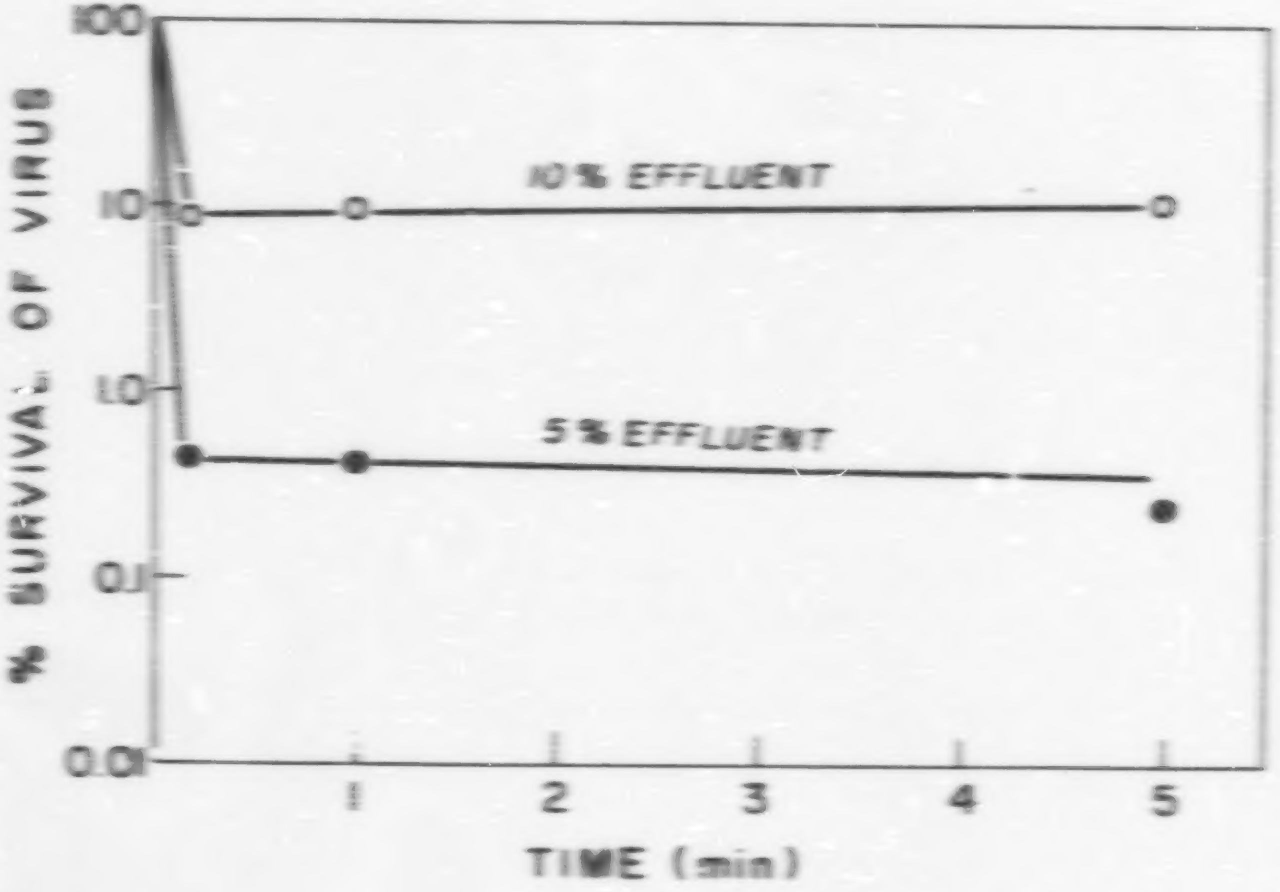


Fig. 28. Inactivation kinetics of poliovirus I by 0.8 mg/L initial O_3 concentration in the presence of 5% and 10% filtered sewage

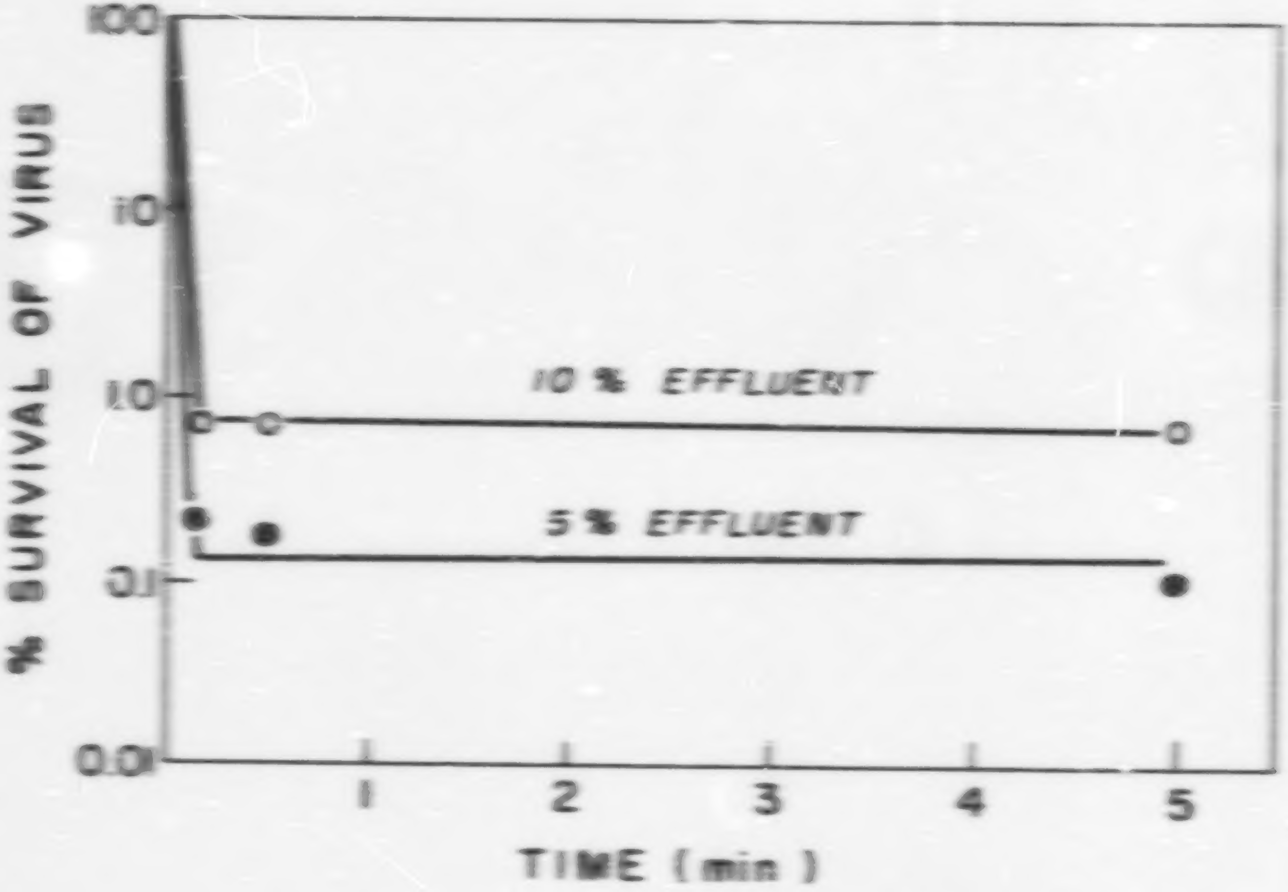


Fig. 29. Inactivation kinetics of poliovirus I by 1.3 mg/l initial O_3 concentration in the presence of 5% and 10% filtered sewage

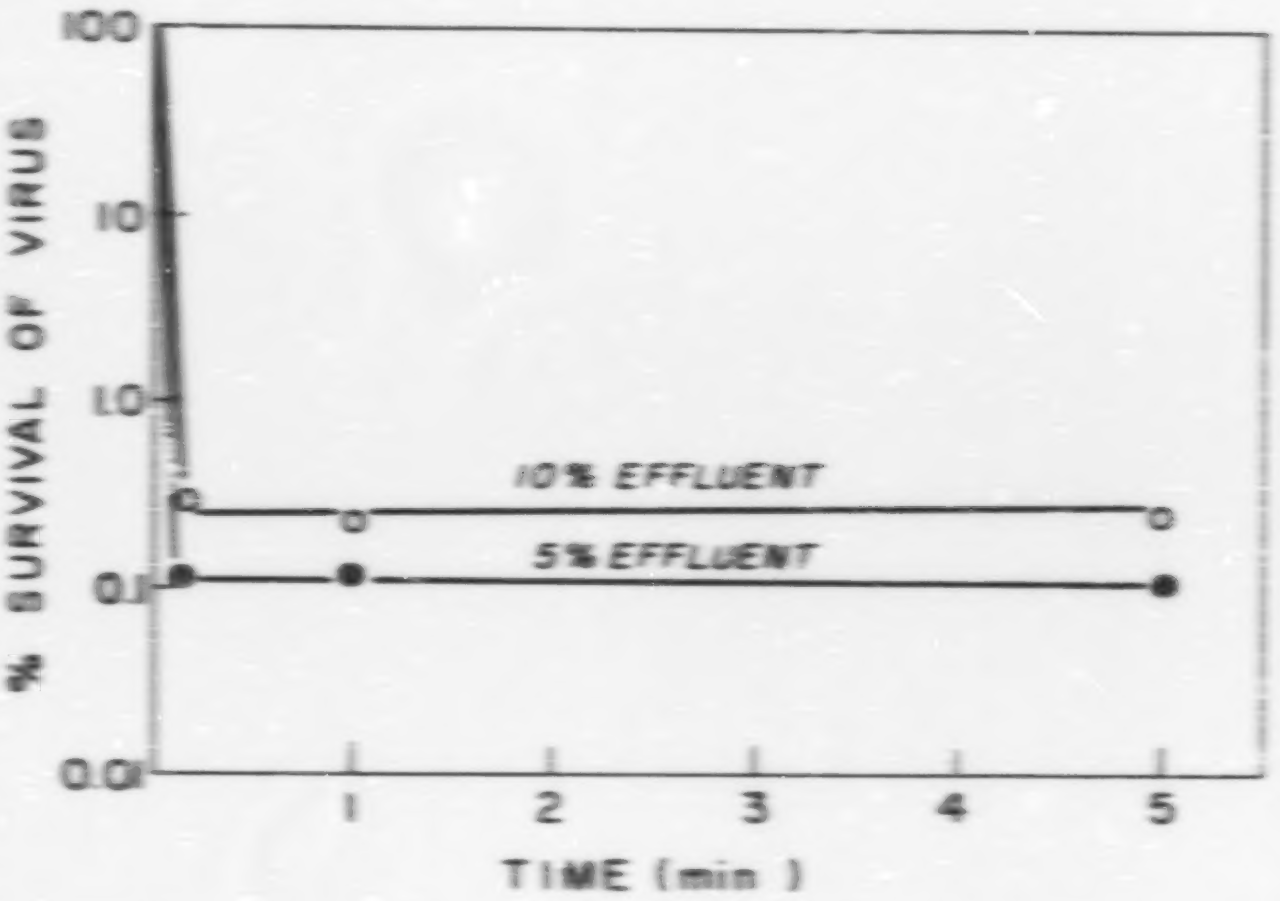


Fig. 35. Inactivation kinetics of poliovirus I by 1.8 mg/l initial I_3 concentration in the presence of 5% and 10% filtered sewage

inactivation took place only during the first ten seconds of the experiment, after which period no significant change in virus titer occurred.

The degree of inactivation correlates with the strength of the O_3 concentration; at concentrations above 1.3 mg/L O_3, however, this correlation disappears, and increasing the concentration causes only slight, if any, increase in the inactivation capacity of O_3. Virus inactivation was also considerably less with the 10% than with the 5% effluent, employing the same O_3 concentration.

Continuous Ozone Bubbling

The virus inactivation obtained with batch experiments was only partially due to the rapid depletion of the residual ozone. Since our objective was to verify whether O_3 would be able to completely inactivate viruses under conditions similar to those in treatment plants, it was decided to introduce ozone in a continuous manner into the filtered sewage. This sewage was either undiluted or diluted 1:2. Virus in ozone-demand-free buffer was used as control. Typical results are depicted in Fig. 31. Ozone residual concentrations in the controls began to rise immediately after bubbling had been started and reached its maximum (3mg/L) in less than three minutes. Simultaneously, the virus titer became sharply reduced, as expressed by the inactivation of 90% in 10 seconds and 99.999% in less than one minute, while the O_3 residual concentration at that time was about 1 mg/L.

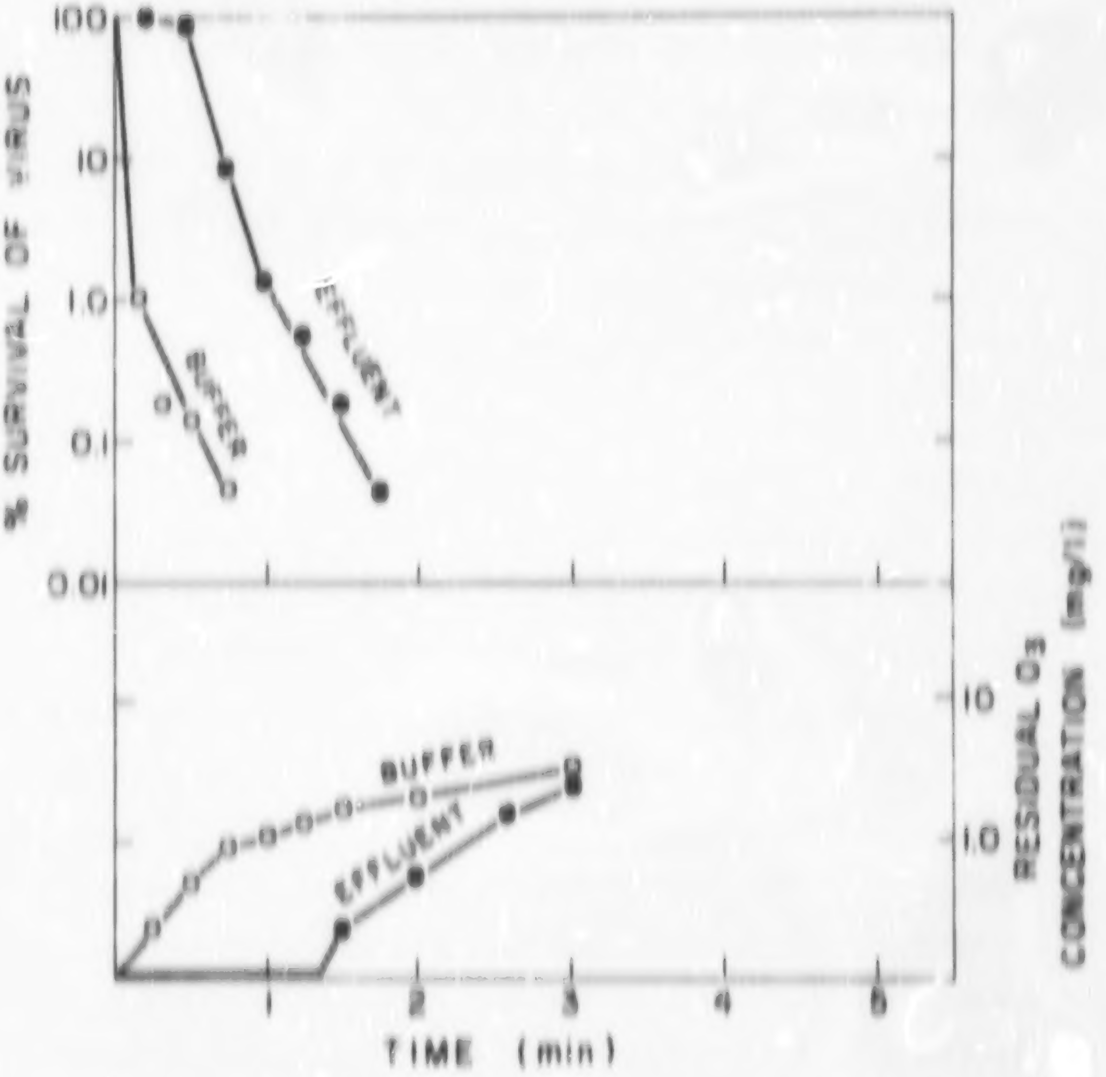


Fig. 81: Kinetics of increase in O_3 residual concentrations and of poliovirus 1 inactivation during continuous O_3 bubbling through buffer and filtered sewage

Ozone residual in the effluent was first detected at 90 seconds after bubbling had been started, after which its concentration increased rapidly. A concentration of 3 mg/L O_3 was reached at the same point in time as noticed with the buffer e.g., about three minutes. Virus inactivation also started after a lag period (30 seconds) of bubbling. Inactivation of 99% of the virus took about one minute from the start of the experiment, at a time when no O_3 residual was detected in the filtered sewage. At two minutes, inactivation of 99.999% of the virus was obtained, while the O_3 residual concentration measured was 0.6 mg/L.

DISCUSSION

Early in the course of ozone experimentation, one encounters the phenomenon of its extreme instability, making it difficult to maintain a constant O_3 level. In a previous study in ozone demand free distilled water[1] special efforts were made to keep a constant O_3 level during experiments designed for inactivation of microorganisms, which allowed for reproducible results. In this earlier study, it was found that very low O_3 concentrations effectively inactivated various microorganisms, among them polioviruses. As to the latter, a high degree of inactivation was obtained with the use of 0.1 mg/L O_3. Coin et al[2] found a threshold value of 0.7 ml/L residual O_3 in surface water samples, below which inactivation was not satisfactory. Their initial O_3 concentration, however, was 5 mg/L. Majumdar et al[3,4] mention a threshold concentration of 1 mg/L O_3 for distilled water.

The main difference between these three latter studies and our previous work is that these authors did not maintain a constant O_3 level. The present study utilized our previous techniques, while employing water possessing ozone demand, which does not allow for constant O_3 residuals. One might therefore expect our results to be more similar to those in the works quoted above.

A further goal of the present study was to test the ability of O_3 to disinfect water in a "true world" situation in which it always shows ozone demand.

With the objective of reproducible results in mind, the same batch of filtered effluent was used throughout the present study. This was achieved by filtering a large quantity of sewage and storing it at -80°C.

A very noticeable occurrence with the batch experiments was the nearly immediate disappearance of the O_3 residual, with partial viral inactivation during this extremely short time interval. The degree of inactivation depended on both the initial O_3 concentration and the quantity of organic matter in the water. Figure 32 depicts the degree of inactivation of poliovirus by different initial O_3 concentrations in 5% and 10% filtered sewage. The amount of organic matter in the effluent played an important role in the ability of O_3 to

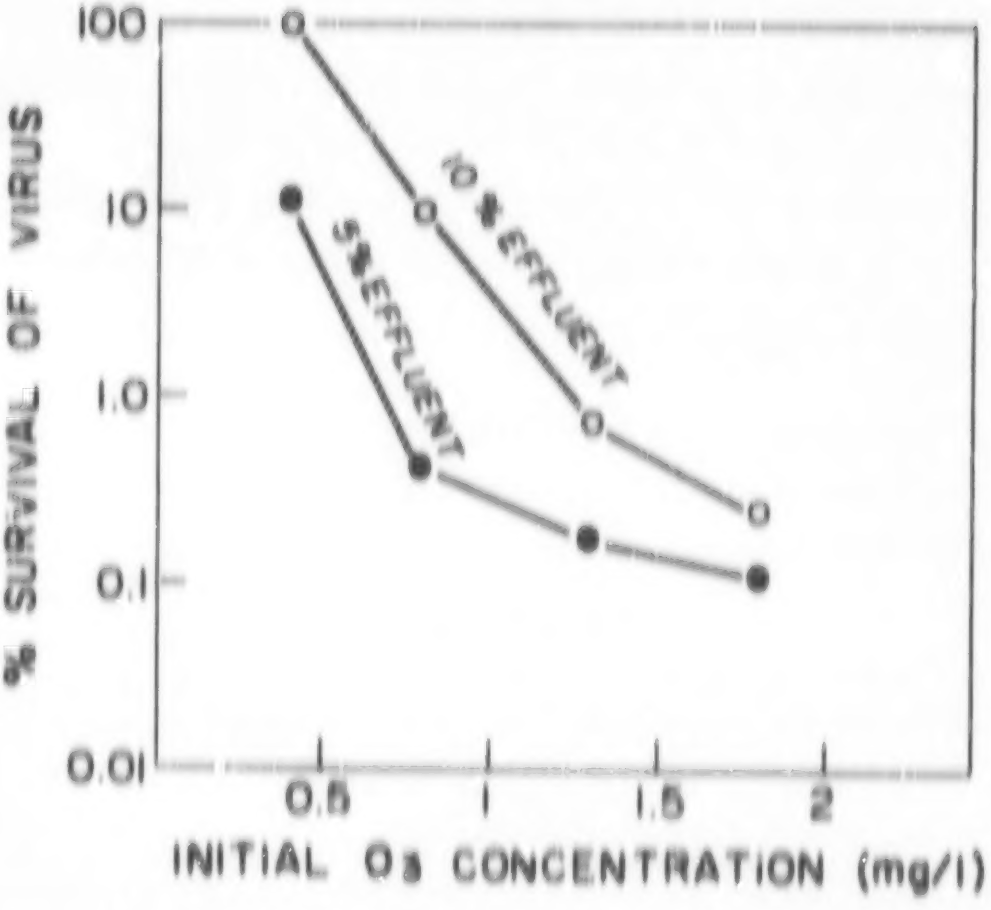


Fig. 32. Percent survival of poliovirus 1 after reaction with different initial O_3 concentrations in 5% and 10% filtered sewage (Batch experiments)

inactivate microorganisms. A large quantity of organic matter (BOD of 50 ppm, COD of 78 ppm, total Nitrogen of 3.8 ppm and Ammonia of .4 ppm) in the liquid impeded the inactivation ability of O_3. This is very clear with low initial O_3 concentrations; increase in O_3 concentration led to a diminished effect of the organic matter. This dose response of O_3 action on viruses was not seen in our previous study with ozone-demand-free water.[1] It is reasonable to assume that O_3 largely reacts with the organic matter, leaving only a small amount to attack the virus. By increasing the initial O_3 concentration, therefore, more O_3 will be available for virus inactivation.

This interference by organic matter with virus inactivation was also observed in the continuous ozone bubbling experiments. In organic-matter free water, detectable O_3 started to appear immediately after the experiment had begun, accompanied by a very rapid virus inactivation. In the presence of organic matter, on the other hand, both the appearance of O_3 residual and the virus inactivation were delayed. Interestingly enough, in the continuous bubbling experiments virus inactivation occurred before the O_3 residual could be detected. A meaningful inactivation of more than 99.9% was achieved at a residual of 0.6 mg O_3/l. The range of O_3 residual concentrations (0.6-1.0 mg/L) which led to a nearly complete inactivation is, in fact, identical to those described by others, also working on systems with ozone demand.[2,3,4]

The overall results attained with ozone show that it has a high potential as a viral disinfectant, even where sewage effluent high in organic content is concerned.

REFERENCES

1. Katzenelson, E., B. Kletter and H.I. Shuval. Inactivation Kinetics of Viruses and Bacteria in Water by Use of Ozone. J.A.W.W.A. 66:725-729, 1974

2. Coin, L., C. Hannoun and C. Gomerria. The Inactivation of the Poliomyelitis Virus Present in Water, By Use of Ozone. La Presse Mediale, 37:72-74, 1964

3. Majumdar, S.B., W.H. Ceckler and O.J. Sproul. Inactivation of Poliovirus in Water by Ozonization. J.W.P.C.F., 45:2433-2443, 1973.

4. Majumdar, S.B., W.H. Ceckler and O.J. Sproul. Inactivation of Poliovirus in Water by Ozonization. J.W.P.C.F., 46:2048-2055, 1974.

5. Shuval, H.I., A. Thompson, B. Fattal., S. Cymbalista, and Y. Wiener. Natural Virus Inactivation Processes in Sea Water. J. San. Engin. Div. 97:587-600, 1971.

6. Schechter, H. Spectrophotometric Method for Determination of Ozone in Aqueous Solution. Water Res. 7:729-739, 1973.

SECTION XIV

COLIPHAGE INACTIVATION IN SEAWATER

INTRODUCTION

Pathogenic bacteria and viruses can be carried by sewage into the sea. It is therefore important to study the inactivation of non-marine microorganisms which enter the marine environment.

Today we have considerable information on the physical, chemical and biological factors[1,2,3] involved in the die-away of enteric bacteria in marine environment. The fate of enteric viruses is much less known.

Previous studies done in our laboratory[4] have shown that there is a definite biological marine anti-viral activity (MAVA) which causes in seven days a three to six log reduction of polio-virus type I seeded into seawater, while in heat-treated seawater, used as control, only a one log reduction was detected.

The MAVA activity was removed from seawater by filtration through a membrane filter with a 0.45 pore size. This activity is also ether sensitive.

The present report is a further study on the factors involved in virus inactivation in seawater. Coliphage T_2 was chosen as a model because working with bacteriophages instead of enteroviruses has the advantage of easy performance of the titration and the short time needed to gain results.

METHODS AND MATERIALS

Assay of Bacteriophage

Samples were diluted in nutrient broth. One ml of each dilution was mixed with 1 ml of 1% liquified agar at 52°C to which 0.1 ml E. coli culture in the logarithmic growth phase was added. After stirring, the mixture was poured on petri dishes containing agar. Plaques were counted after 18 hours incubation at 37°C.

Isolation and Counts of Bacteria from Seawater

Seawater samples were taken from the Palmachim Beach 30-40 meters from shore, 80 cms. depth. No known source of pollution exists near the sampling point. This location can be considered as characteristic for an unpolluted area of the Eastern Mediterranean. The samples were inoculated on Zo Bell medium 2216 and incubated at 20°C. The formula of the medium was as follows: 0.5 gr Bacto peptone, 1.0 gr Bacto yeast extract, 0.2 gr sodium thiosulfate, 1,000 ml seawater (pH of the mixture after autoclaving 7.4). Descrete colonies which grew on the media, were picked and subcultured on agar slopes of Zo Bell media. All pure cultures were stored at 4°C. Counts of marine bacteria were done by diluting the sample in sterile seawater. 0.1 ml of the dilution of the original sample were spread with a bent glass rod on Zo Bell agar. The plates were incubated at 20°C.

Assays for MAVA in Seawater

Ehrlenmeyer flasks of 250 ml, containing 50 ml seawater, were inoculated with 2.5×10^8 coliphage particles suspended in 1 ml phosphate buffer pH 7.5 (final bacteriophage concentration 5×10^6 particles/ml). The inoculated seawater was incubated at 20°C. Counts of phage and of marine population were made immediately after inoculation (0 time) and afterwards in intervals of 24-48 hours.

Sterilization of Seawater

Seawater was sterilized by filtration through a Sartorius membrane filter of 0.45μ pore size.

Washing of Marine Bacteria

The bacteria were washed by centrifugation. A bacterial suspension in sterile seawater was centrifugated 10,000 rpm for 10'. The sediment was resuspended in sterile seawater and centrifugated again. This cycle was repeated five times.

RESULTS

Assays for Anticoliphage Activity in Seawater

Studies of the anticoliphage activity of seawater were carried out under laboratory conditions. An inactivation curve of coliphage in fresh seawater samples is shown graphically in Figure 33. The inactivation process involves two stages. A lag phase lasting 4 to 8 days after which a decrease in the titer occurs.

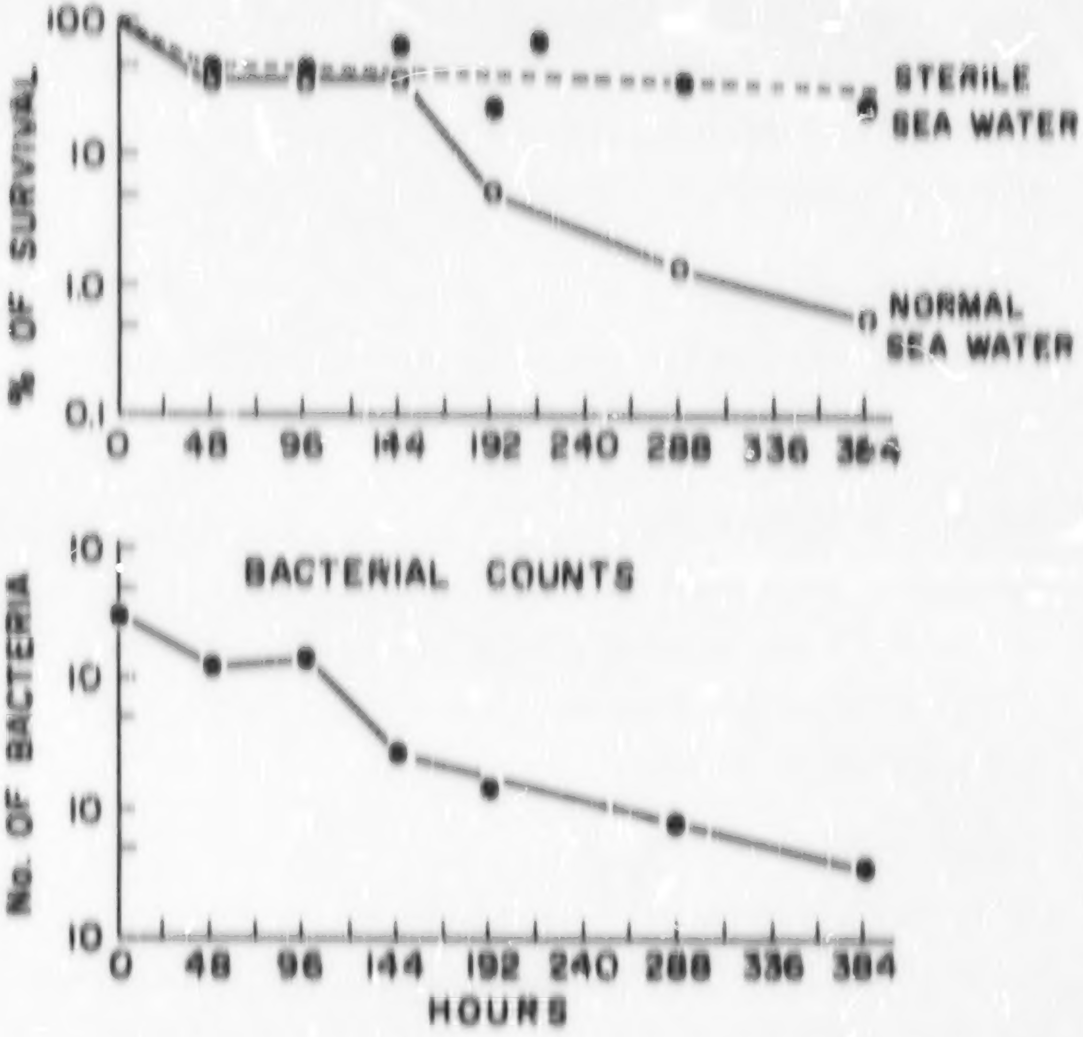


Fig. 33. Inactivation of coliphage T_2 in normal and autoclaved sea water

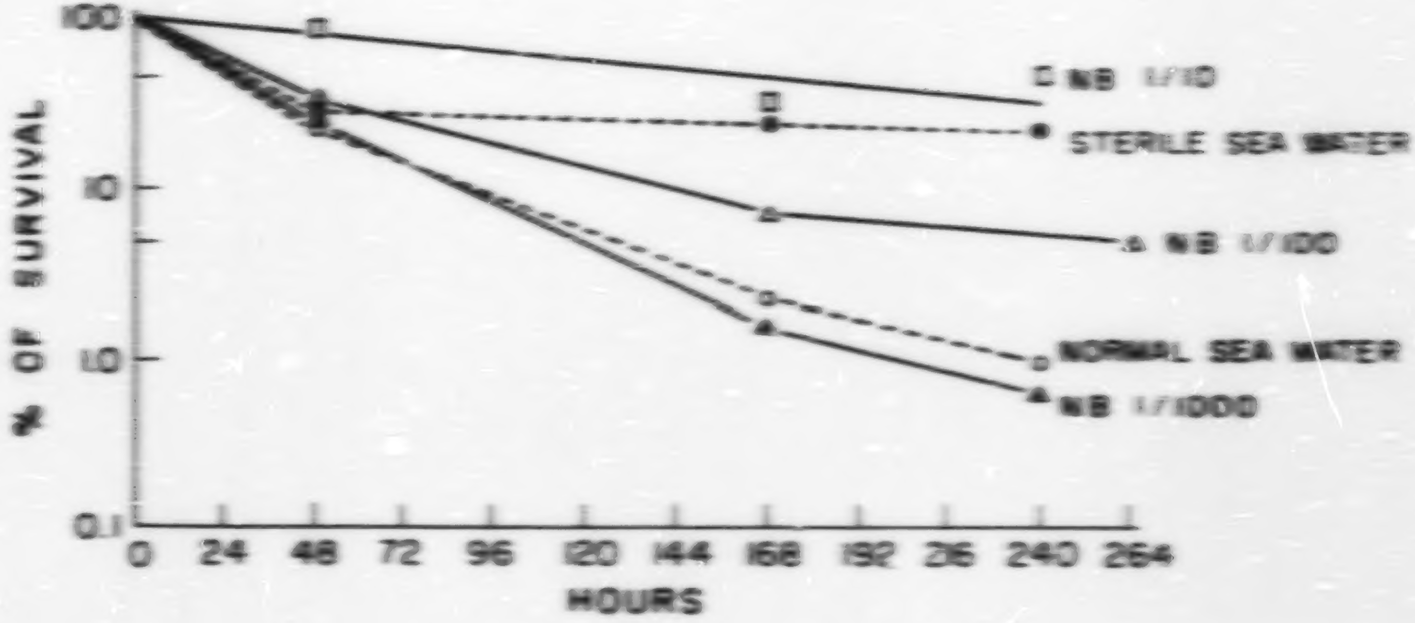


Fig. 3A. Inactivation of coliphage T_2 in sea water containing different concentrations of nutrient broth

In experiments in which marine bacteria were suspended in sterile seawater and in concentrations of 10^7 bacteria/ml the decrease in phage titer took place without any lag phase (Figures 35, 36, 37 and 38).

The initial number of marine bacteria in seawater ranged from 10^4-10^5 bacteria per/ml (Fig. 34 bottom). This number decreased during the assay period in 1-2 logs.

The Effect of Organic Matter on the Anticoliphage Activity in Seawater

To study the effect of added organic matter on the self-purification processes in seawater, assay for anticoliphage activity were performed in seawater containing different amounts of nutrient broth. Figure 34 shows that addition of organic matter to seawater inhibits its anticoliphage activity of normal seawater. Figure 35 shows graphically the effect of nutrient broth on the anticoliphage activity of a mixture of serially washed marine microorganisms added to sterile seawater in the concentration of 10^7 bacteria/ml. Addition of 0.3% nutrient broth powder inhibited the anticoliphage activity of the microorganisms.

Anticoliphage Activity of Isolated Marine Microorganisms

Five different pure cultures of marine bacteria in the stationary growth phase were washed and suspended in sterile seawater in the concentration of 10^7 bacteria/ml. The anticoliphage activity of these isolates is depicted in Figure 36. Three of these isolates showed anticoliphage activity. The isolates having anticoliphage activity differed widely in the morphological characteristics of their colonies. All of them

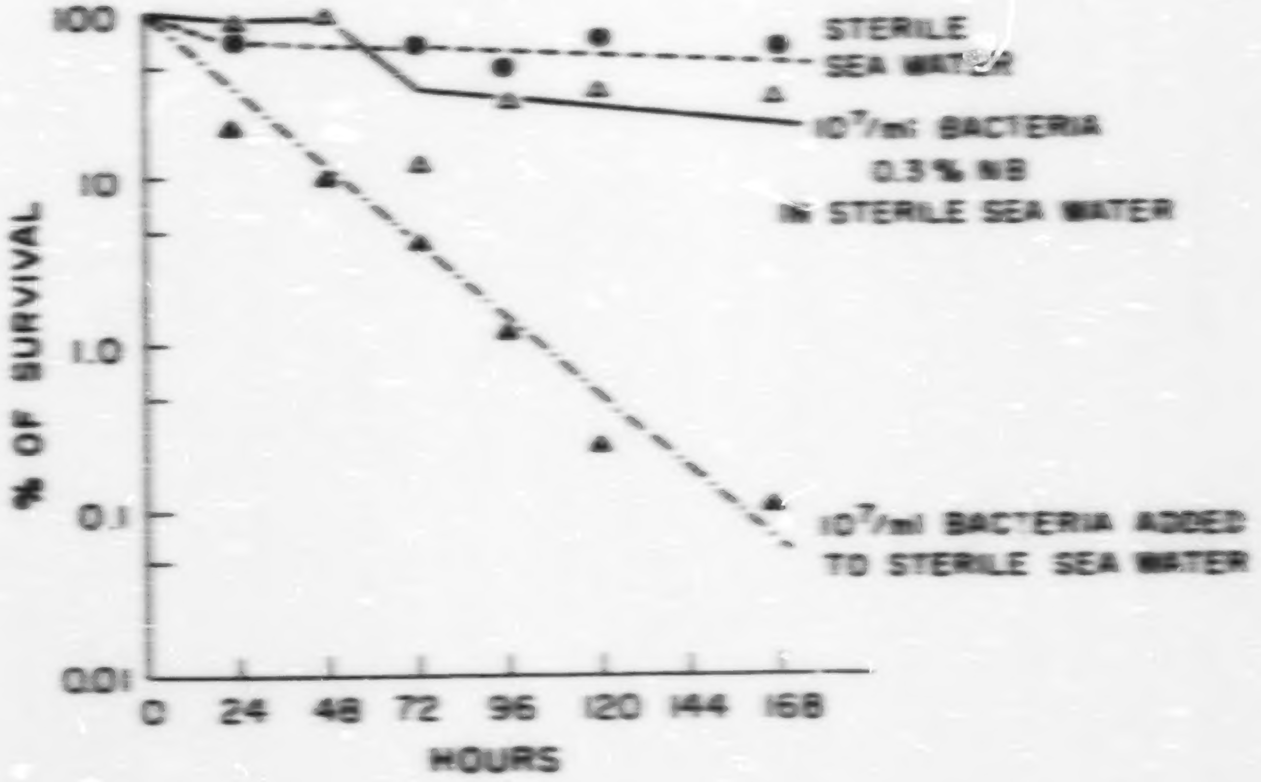


Fig. 35. The effect of nutrient broth on the anticoliphage activity of a mixture of marine bacteria

were Gram negative. One isolate was a vibrio and the two others were rods of different sizes.

The positive cultures isolated in this study showed reduced activity when assayed again after several transfers on Zo Bell medium and stored at 5°C for three months. Figure 37 shows the inactivation activity of the same bacteria (isolate IV) suspended, in the first case, in sterile seawater immediately after isolation and in the second case, after several transfers on Zo Bell medium and storage at 4°C for 3 months.

In a separate experiment another marine bacterium was isolated. This organism showed identical activity to MAVA but unlike the previous isolate, did not lose its activity when transferred on Zo Bell medium. The bacterium is apparently a Flavobacterium sp. It grows well on Zo Bell medium at 20°C; at 37°C however, it does not multiply.

The Effect of Organic Matter on the Anticoliphage Activity of Isolates of Marine Bacteria

To examine some factors in seawater that might reduce the rate of coliphage inactivation by marine bacteria, 0.3% nutrient broth powder was added to a suspension of marine bacteria (isolave IV) in concentration of 7×10^6 bacteria/ml in sterile seawater. This suspension was assayed for anticoliphage activity. The same isolate, suspended in sterile seawater without nutrient broth, was used as a control. Figure 38 shows that addition of nutrient broth inhibited the antiphage activity of the marine bacteria, although it stimulated growth of the bacterium.

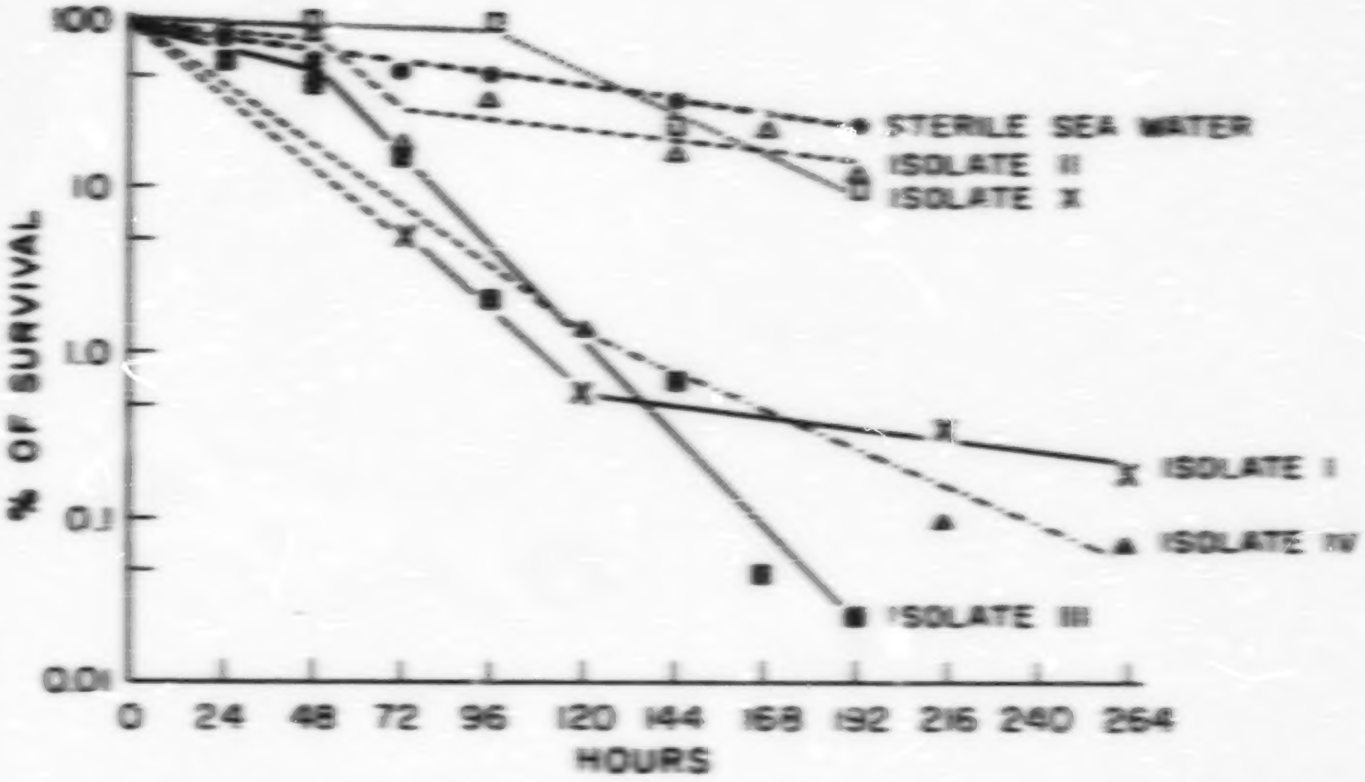


Fig. 36. Anticoliphage activity in different isolates of marine bacteria

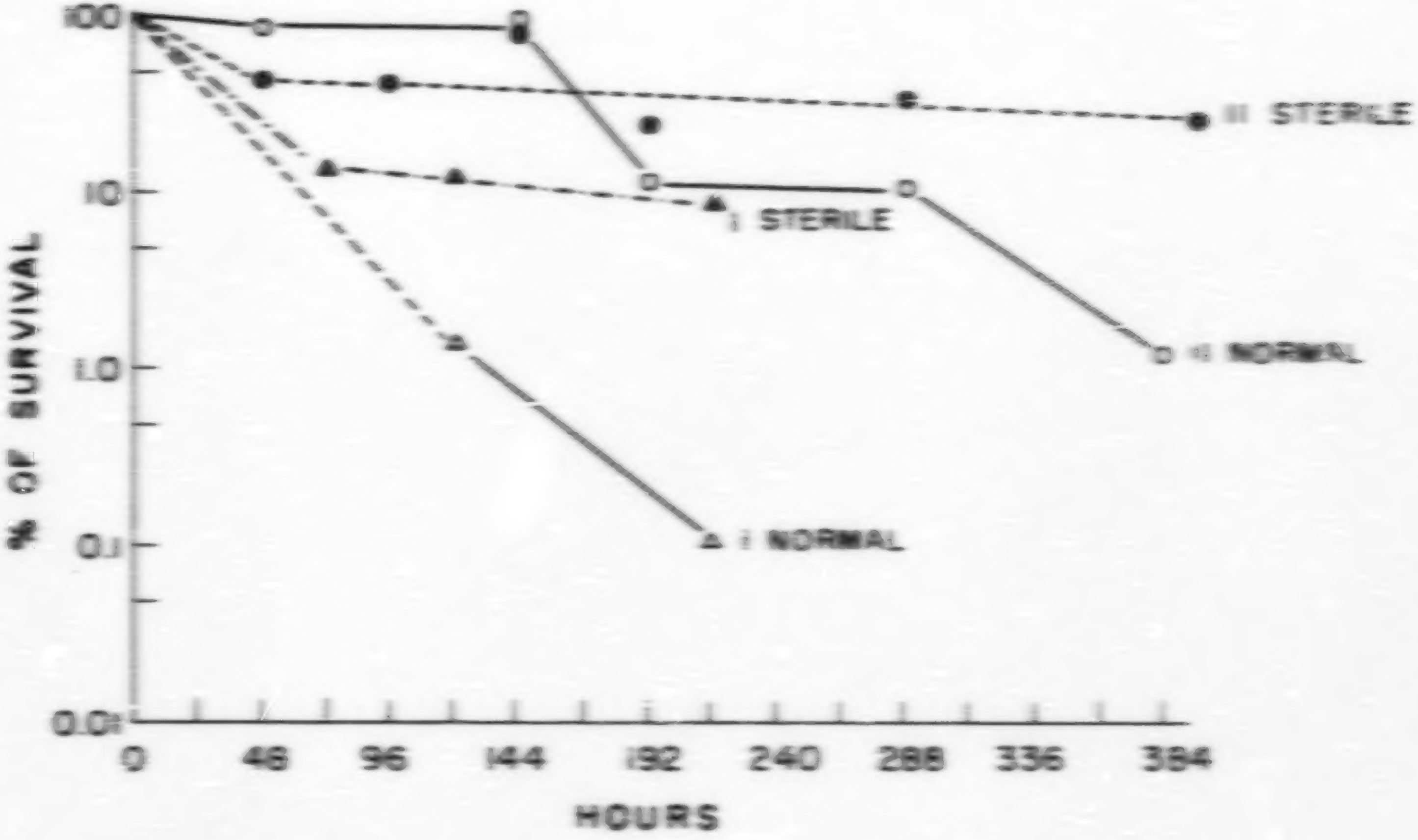


Fig. 37. Different rates of coliphage inactivation by an isolate of marine bacteria

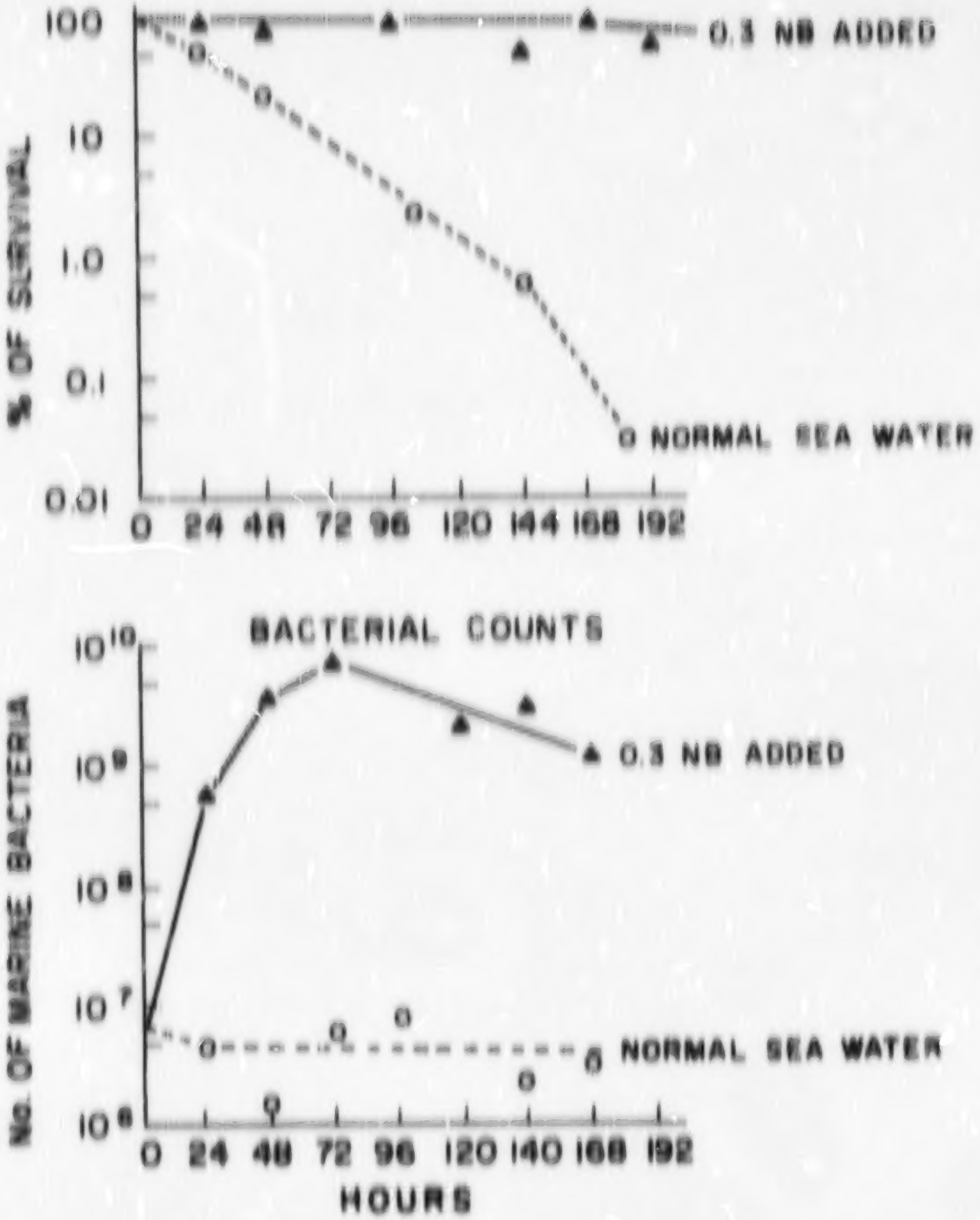
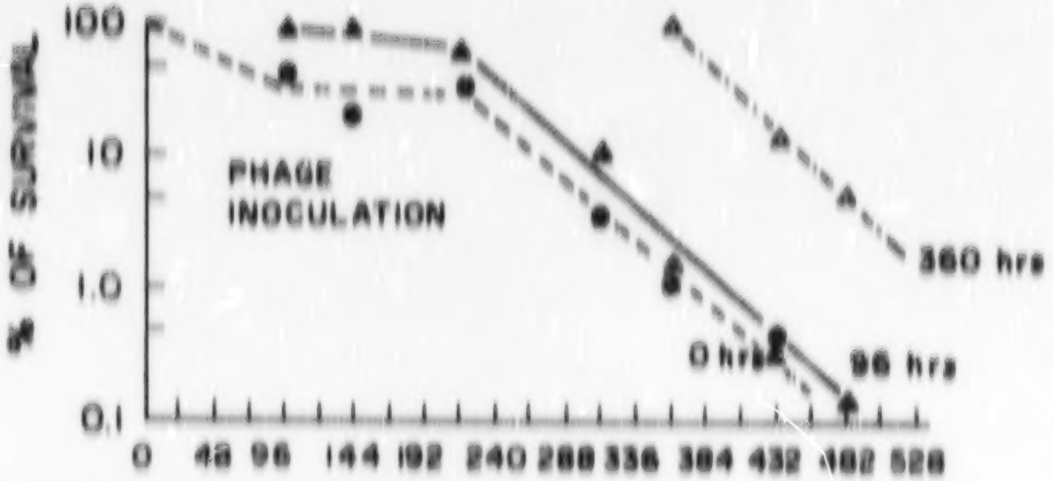


Fig. 38. The effect of nutrient broth on the activation capacity of an isolated marine bacteria



BACTERIAL COUNTS

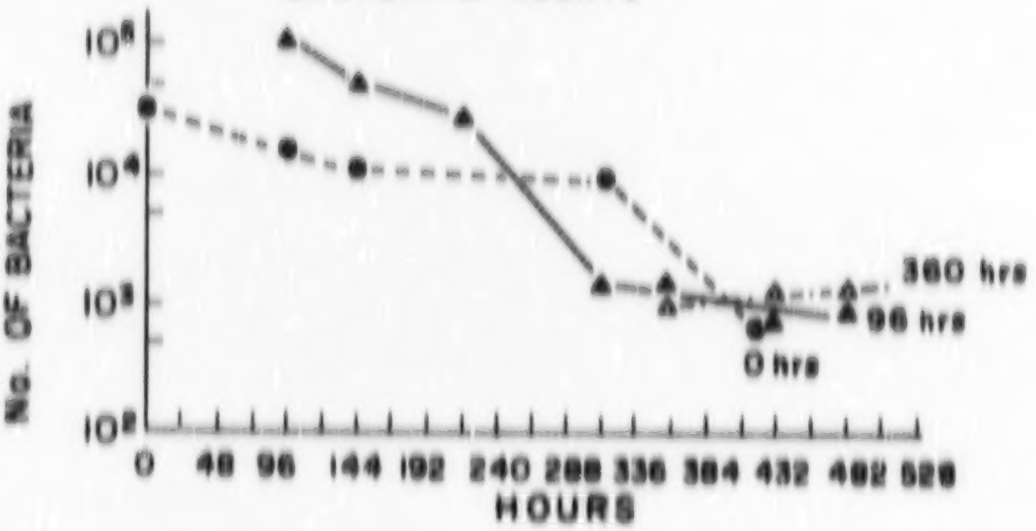


Fig. 39. The inactivation of coliphage in preincubated sea water and the indicated hours of preincubation in 20°C before inoculation of the phage

The Effect of the Physiological State of the Marine Bacteria on Their Anticoliphage Activity

Bacterial counts in seawater to which coliphage were added showed that the coliphage inactivation was associated with a decrease in the marine bacterial population (see Figure 38) and it usually occurred after a lag period. In order to study the effect of this phenomena on coliphage inactivation in seawater, the following experiments were performed. Several Erlenmyer flasks containing seawater were incubated at 20°C in the dark. Each flask was inoculated with coliphage at a different time interval after incubation had begun. The rates of coliphage inactivation in the pre-incubated seawater are summarized in Figure 39. The inactivation curve of the coliphage, inoculated in seawater pre-incubated for 360 hours, lacks the lag period which characterized the inactivation in fresh seawater. In that suspension the bacterial population was already in the decline phase at the time the phage was introduced.

DISCUSSION

In this report some factors involved in the self-purifying capacity of vitro seawater were studied. The coliphage inactivation model was chosen because T bacteriophages, like enteroviruses, are found in domestic sewage and are discharged into seawater.

Mitchell[6] noted that the antiviral activity of natural seawater was stronger than that of seawater polluted with organic matter. Lycke et al[6] described a heat sensitive antiviral factor in seawater. Proteins and amino acids were found to inhibit this factor. They suggested that the inhibition was caused by reaction of the proteinious material with the antiviral factor.

Our observations correlate quite well with those mentioned above. Addition of nutrient broth inhibited the antiviral activity of seawater. The inhibitory effect of nutrient broth could be caused by its action on either the marine microorganisms or on their products.

Phages can be used as a source of food for bacteria when they are digested by proteolytic enzymes produced by the bacteria. Such a possibility was described by Cliver and Herrmann[7] who showed that proteolytic enzymes of Pseudomonas aeruginosa can be involved in nutrition or autolysis. Nutrient broth may protect the bacterio-phages by competitive inhibition.

A loss of the antiviral activity by marine isolates after several transfers and storage was also observed by other authors. Magnusson et al[8] isolated a marine microorganism with a virus inactivating capacity. This capacity was lost after several transfers on artificial media and storage at 25°C. We suspect that nutrients, which had been stored in the bacteria during the passages on rich media, caused the loss of phage inactivating activity. This may also be the reason for the reduced antiphage activity in some of our experiments.

Studies on the kinetics of coliphage inactivation in pre-incubated seawater showed that the lag phase was lost after a long pre-incubation of natural seawater at 20^{0}C. During that time, the number of marine bacteria decreased at least in one lag. It is possible that during this period bacterial exocellular polymers were produced and these caused phage flocculation or absorption, thus decreasing the number of phages in the suspension. Such exocellular polymers were shown by Pavoni et al.[9]

SUMMARY

Seawater has been shown to possess a self-purifying capacity which enables it to inactivate foreign microorganisms. The present report deals with biological and chemical factors involved in the inactivation of coliphage T_2 in seawater. The aims of this study were:

1. Study of kinetics of T_2 phage inactivation in seawater.
2. Isolation of marine microorganisms involved in phage inactivation.
3. Investigation of factors that inhibit the antiviral activity in seawater.

The main findings were:

1. A typical inactivation curve of T_2 phage in fresh seawater involved two stages: a lag phase after which a decrease in the titer occurs.
2. When marine bacteria were added to sterile seawater the decreases in phage titer occurred without a lag phase.
3. Some marine microorganisms were isolated and their anticoliphage activities in sterile seawater was examined.

4. Nutrient broth in low concentration was found to inhibit the antiviral activity of seawater.

5. Pre-incubated seawater exhibits the anticoliphage activity without any lag period.

6. Two alternative explanations for the anticoliphage activity in seawater were suggested: a) phage are digested by proteolytic enzymes of marine bacteria, and b) bacterial excellular polymers cause biological flocculation.

REFERENCES

1. Magnusson, S., C.E. Hedstrom, and E. Lycke. The Virus Inactivation Capacity of Seawater. Acta Path. Microbiol. Scand. 66:551-559, 1966.

2. Matossian, A.M. and G.A. Garabedian. Virucidal Action of Seawater. Amer. J. Epidemiol. 85: 1-8, 1967.

3. Mitchell, R. Factors Affecting the Decline of Nonmarine Microorganisms in Seawater. Water Res. 2:535-543, 1968.

4. Shuval, H.I., A. Thompson, B. Fattal, S. Cymbalista and Y. Wiener. Natural Virus Inactivation Processes in Seawater. J. Sanitary Eng. Div. 97:587-600, 1971.

5. Mitchell, R. and H.W. Jannasch. Processes Controlling Virus Inactivation in Seawater. Environ. Sci. Technol. 3:771-773, 1969.

6. Lycke, E., S. Magnusson and E. Lund. Studies on the Nature of Virus Inactivating Capacity of Seawater. Arch. Ges. Virus. Forsch. 17:409-413, 1966.

7. Cliver, D.O. and J.E. Herrmann. Proteolytic and Microbial Inactivation of Enteroviruses. Water Res. 6:797-805, 1972.

8. Magnusson, S., K. Gunderson, A. Brandberg, and E. Lycke. Marine Bacteria and their Possible Relation to the Virus Inactivation Capacity of Seawater. Acta Path. Microbiol. Scand. 71:274-280, 1967.

9. Pavoni, J.L., M.W. Tenney and W. F. Echelberger. Bacterial Exocellular Polymers and Biological Flocculation. J.W.P.C.F. 44:414-431, 1972.

LIST OF PUBLICATIONS

1 Shuval, H.I. and E. Katzenelson, "The Detection of Enteric Viruses in the Water Environment" in: *Water Pollution Microbiology*, (ed.) R. Mitchell, John Wiley, New York, pp 347-381, 1972.

2. Shechter, H., "Spectrophotometric Method for Determination of Ozone in Aqueous Solutions", *Water Res.* Pergamon Press, Vol. 7, pp. 729-739, 1973.

3. Katzenelson E., B. Kletter, Hanna Schecter and H.I. Shuval, "Inactivation of Viruses and Bacteria by Ozone", in: *Chemistry of Water Supply, Treatment and Distribution*, (ed.) A.J. Rubin, Ann Arbor Science Publishers, pp. 409-421, 1974.

4. Katzenelson, E., B. Kletter and H.I. Shuval, "Inactivation Kinetics of Viruses and Bacteria in Water by Use of Ozone", *Jour. American Water Works Association*, Vol. 66, pp. 725-729, 1974.

5. Fattal, B., E. Katzenelson and H.I. Shuval, "Comparison of Methods for Isolation of Viruses in Water", in: *Virus Survival in Water and Wastewater Systems*, (eds.) Malina and Sagik, Center for Research in Water Resources, The University of Texas at Austin, pp 19-30, 1974.

6. Belfort, G., Y. Rotem and E. Katzenelson, "Virus Concentration Using Hollow Fiber Membranes," *Water Res.*, Vol. 9, pp. 79-85, 1974.

7. Katzenelson, E., "A Rapid Method for Quantitative Assay of Poliovirus from Water with the Aid of the Fluorescent Antibody Technique", *Archives of Virology*, Vol. 50, pp. 197-206, 1976.

8. Belfort, G., Y. Rotem and E. Katzenelson, "Virus Concentration Using Hollow Fiber Membranes,II", *Water Res.* Vol. 10, pp. 279-284, 1976.

9. Kletter, B. and E. Katzenelson, "Coliphage Inactivation in Seawater", *Acta Adriatica*, 1976, accepted for publication (15 pages).

10. Katzenelson E. and N. Biedermann, "Disinfection of Viruses in Sewage by Ozone", *Water Res.*, 1976, accepted for publication (10 pages).